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RESPIRATION IN THE CENTRAL NERVOUS SYSTEM

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Effects of oxygen lack on the central nervous system. The study of respiratory processes in the central nervous system is of more than ordinary interest. This is not only because metabolism in the brain presents a number of unique features, but because there appears to be a close association between oxidation processes in the brain and cerebral activity.

It is known that the gray matter of brain has a high rate of oxygen consumption and that a continuous supply of blood to the brain is essential for the normal functioning of this organ. An interruption of cerebral circulation for six to eight seconds will, for example, produce loss of consciousness. The usual action potentials in the cat's cortex are obliterated by a cerebral anemia of twenty seconds (Simpson and Derbyshire, 1934) and an anemia varying from two to fifteen minutes frequently causes irreversible changes in the cerebral cortex and medulla. The effects of cerebral anemia have been frequently described (e.g., Gildea and Cobb, 1930) and the valuable review of Wolff (1936) on the cerebral circulation gives much useful information on the consequences of decreased oxygen supply to the brain.

Lennox, Gibbs and Gibbs (1935) studied the cerebral blood flow and oxygen saturation of the blood returning from the brain in twenty-two unanesthetized human subjects with reference to loss of consciousness due to 1, syncope; 2, breathing of nitrogen; 3, pressure on the carotid sinus. Oxygen saturation of the blood leaving the brain through the internal jugular veins was measured. Their results make it clear that unconsciousness supervenes if the oxygen supply to the brain is suddenly reduced to such an extent that oxygen saturation of the blood in the internal jugular vein falls to 24 per cent or less. With a saturation value of 30 per cent or more the person remains conscious. It was also found that pressure on the carotid sinus brings about unconsciousness

without a preliminary cerebral anemia or fall in cerebral blood flow. There is no decrease in total cerebral blood flow during sleep (Gibbs, Gibbs and Lennox, 1935) or in epileptic seizures (Lennox and Gibbs, 1936; Gibbs, Lennox and Gibbs, 1934). It is evident, therefore, that although the unconscious state may take place as a consequence of lowering the oxygen supply to the brain it may also occur when oxygen is as freely available to the cerebral tissues as in the conscious condition.

Lennox (1936) impressed with the importance of an abundant oxygen supply to the brain thought it probable that the brain would be amply supplied with capillaries, since "the number of capillaries per unit of tissue is usually a gauge of the metabolic activity of that tissue" (see also Wolff, 1936), and that blood leaving the brain would remain rich in oxygen. It had been shown, however, by Cobb and Talbot (1927) that the number of capillaries of gray matter is small compared with that of muscle, and Lennox found that the residual oxygen saturation of the blood in the internal jugular vein on leaving the brain in conscious humans was approximately 60 per cent. This value was definitely lower than the oxygen content of venous blood from the arm (72 per cent). This points either to a relatively high consumption of oxygen by the tissues of brain, or to a smaller rate of flow of blood through the brain. Wolff (1936), however, considers that the brain circulation time does not differ essentially from that of other organs. Lennox further showed from a study of the distribution of the values of the oxygen saturation of samples of blood from the internal jugular vein, from a vein in the arm and from the femoral vein, that the speed of blood flow in the brain is more constant than in the extremities and suggests that a constant supply of oxygen to the brain is of greater importance than a rich supply (see also Cobb and Forbes, 1937). Acute anoxemia, it is pointed out (Lennox, 1936), may produce nervous symptoms not observed in states of chronic oxygen lack. While it is probably quite true that a constant, rather than a rich, supply of oxygen to the brain is essential to the normal functioning of this organ, it may be suggested that this is related to the fact that the brain is very restricted in its choice of metabolites for respiratory purposes. As will be shown later the brain makes its chief demand for respiratory activity on glucose. Hence a constant respiratory activity of the brain will depend on the availability of at least a minimum concentration of glucose and on a constant supply of oxygen. It is evident that the respirations of organs which have a "mixed" metabolism will fluctuate very considerably with changes in the concentrations of the various metabolites

attacked by these organs and it seems unlikely that variations of oxygen tension will produce such serious consequences in the functioning of these organs as in the case of brain which is so much more restricted in its choice of metabolites.

Experiments in which the circulation of dogs is completely stopped and, after varying intervals, re-initiated have been carried out by Heymans and Bouckaert (1935). After 30 minutes of anoxemia neurones in the brain can still regain their function (respiration, vasomotor and pupillary reflexes). Oxygen lack longer than five minutes results in the cessation of cerebral function but within this interval full recovery (except for blindness) can take place. Various parts of the nervous system differ obviously in their sensitivities to oxygen lack and abilities to recover.

It has been shown by Lennox that the seizures of epileptic patients can be regularly induced by administration of air deficient in oxygen. An attack of petit mal would occur when the oxygen tension of the respired air had fallen to 8–12 per cent whereas unconsciousness does not supervene in normal persons until the oxygen content is reduced to 7 per cent. Conversely an increased oxygen pressure will diminish the number of seizures in an epileptic patient (Lennox and Behnke, 1936), but it is to be noted that high oxygen pressures will themselves induce convulsions. This had already been noted by Bert in 1878, and Shilling and Adams (1933) found that rats confined to oxygen filled chambers would all convulse if the oxygen pressure was raised to $4\frac{1}{2}$ atmospheres. No convulsions took place at a pressure of 3 atmospheres.

It is known that under certain conditions of respiratory depression the administration of oxygen may further depress respiration, probably by removal of an anoxemic stimulus (e.g., CO_2) to respiration. Information concerning these phenomena, a description of which is not strictly relevant to the subject of this review, may be found in the papers of Loevenhart and his colleagues (1918, 1922), d'Elseaux and Solomon (1933) and Marshall and Rosenfeld (1936).

Psychological consequences of oxygen lack. Many of the consequences—both physiological and psychological—of oxygen want have been described by Barcroft (1934, 1936), and by McFarland(1932, 1937) and will not be considered here in any detail. Reference may be made, however, to the recent work of McFarland, Knehr and Berens (1937) who have shown that under anoxemia there is a decrease in efficiency of ocular movements. This is attributed to a diminished amount of oxygen being delivered to the nervous tissue, subcortical as well as

cortical. They consider that the changes in eye movements can be used to detect early effects of oxygen deprivation. Gellhorn (1936) has studied the influence of administration of 3 per cent CO_2 on the effects of oxygen deficiency on visual intensity discrimination. Oxygen deficiency (breathing 8 per cent–9 per cent oxygen) produces a marked decrease in visual discrimination and this is completely offset by 3 per cent CO_2, which itself has no effect on this sensory function. Gellhorn considers that the alleviating effect of the CO_2 is due to the circulatory improvement induced by CO_2 under oxygen deficiency. He points out (Gellhorn, 1937) that 3 per cent CO_2 improves oxygenation of the tissues in general and of the brain in particular by

1. An increase in the respiratory volume.
2. The right shift in the oxygen dissociation curve (Barcroft, 1928).
3. The improved venous return due to the increased muscular tonus (Henderson *et al.*, 1936).
4. The specific dilatory action of CO_2 on the blood vessels of the brain (Cobb and Fremont-Smith, 1931; Gibbs *et al.*, 1935b).
5. The augmentation of rise in blood pressure which occurs under conditions of oxygen want (Lambert and Gellhorn, 1937).

It is interesting to note the fact (Gellhorn and Joslyn, 1936) that oxygen deficiency (breathing 7 per cent–9 per cent O_2), hyperpnea and carbon dioxide excess (6 per cent–7 per cent CO_2) have marked prolonging effects on the time required to accomplish simple mental tests such as number cancellation and the Kraepelin additions test. The results are explained on the basis of insufficient oxygen supply to the cortex. Kraines (1937), too, has shown that oxygen deficiency (breathing 10 per cent O_2) leads to a diminution of intellectual function.

Oxygen consumption by brain in vivo. The data for determinations of the oxygen uptake of brain *in vivo* are not very accurate owing to the obvious experimental difficulties of ascertaining the amount of blood going to the brain, etc. Some approximate estimations may be given.

Himwich and Nahum (1932), recalculating the results of Alexander and Cserna, find that 13 ml. oxygen are consumed per 100 grams dog brain per minute. This gives a Q_{O_2}[1] (μl. O_2 consumed per milligrams dry weight of tissue per hour) of 39 $\left[\text{i.e., } \frac{13000 \times 60}{20000}\right]$ taking the dry weight of brain to be 20 per cent of the wet weight. Hou (1926) calculated that per 100 grams dog brain 5.6 to 7.8 μl O_2 were utilized per minute.

[1] This value, following Warburg, is usually negative since it indicates an absorption of O_2. The negative sign will, however, be omitted in this article.

This gives a Q_{O_2} of 16.8 to 23.4. From values quoted in Page's *Chemistry of the Brain*, p. 326, the Q_{O_2} of rabbit brain *in vivo* is 28.2 (Yamakita) and of dog brain 29.7 (Gayda).

According to Lennox (1936) the oxygen content of blood leaving the human brain is about 60 per cent saturation and is less than that of blood leaving the face and the extremities. The following table 1, taken from his paper, shows his measurements.

There would appear to be a loss of approximately 8 ml. O_2 per 100 ml. of blood in its passage through the human brain. The estimated flow

TABLE 1

Average gaseous composition of blood leaving the face, brain, arm and leg of man

VEIN	O_2 CONTENT PER CENT BY VOLUME	O_2 CAPACITY PER CENT BY VOLUME	O_2 PER CENT SATURATION	CO_2 CONTENT PER CENT VOLUME
Internal jugular	12.0	19.4	61.5	54.7
Antecubital	13.0	20.3	64.2	52.8
Femoral	13.3	19.2	69.2	52.6
External jugular	17.8	20.5	87.0	51.2

of blood through a human brain is about one liter per minute (Schneider and Schneider, 1934)[2]. Taking the weight of the brain as 1400 grams the Q_{O_2} is

$$\frac{80{,}000 \times 60}{280 \times 1000} = 17.1$$

These figures are given to show the order of magnitude of Q_{O_2} of brain *in vivo;* they cannot be regarded as accurate.

Respiratory quotient of brain in vivo. The respiratory quotient of brain *in vivo* is approximately unity. Lennox (1931) compared the O_2 and CO_2 contents of arterial blood with those of the blood in the internal jugular vein, and obtained an R. Q. of 0.95. This quotient is definitely higher than that of the arm (0.86) and of the leg (0.72). Lennox pointed out the limitations of determinations of the respiratory quotient by the arterio-venous difference. The CO_2 content of the blood depends not only on changes in tissue metabolism but on variations in respiratory rate, on the rate of diffusion of CO_2 through the skin and on the acid base composition of the blood. Induced hyperpnea

[2] The flow through rabbit brain is 60 ml. per 100 grams per minute (Winterstein, 1935).

and breathing CO_2 bring about apparent changes in the R. Q. of brain and Lennox arrived at an average R. Q. of 0.95 for human brain on the basis of a large number of experiments. Himwich and Nahum (1932) examined a series of dogs, some of which were anesthetized with amytal, others phlorizinized and some depancreatized and found the R. Q. of the brain *in situ* to be unity.

Such a respiratory quotient points to the probability that carbohydrate is the main fuel of the brain *in vivo*. Myerson and Halloran (1930, 1931) and Lennox (1931) have shown that blood on passing through the brain loses carbohydrate. The difference between the glucose contents of arterial and cerebral venous blood (9 mgm. per cent) is greater than that between the glucose contents of arterial and muscle venous blood (arm 5 mgm. per cent, leg 4 mgm. per cent). Himwich and Fazekas (1937) have examined cerebral blood samples from the superior longitudinal sinus and arterial samples from the femoral arterial. They find the average difference between the glucose contents of these arterial and venous bloods to be 13 mgm. per cent, the animals used being dogs anesthetized with amytal.

If we take the value 10 mgm. per 100 ml. blood to be a roughly accurate measure of the glucose consumption of human brain *in vivo*, then the amount of oxygen required to burn this amount of glucose completely to CO_2 and H_2O would be 7.5 ml. (at N.T.P.). This value corresponds fairly closely to the fall in oxygen content per 100 ml. blood on passing through the brain (see table 1). Again in the experiments of Himwich and Fazekas (1937) the glucose consumption by the brain of amytalized dogs is 13 mgm. per 100 ml. blood. This corresponds to an oxygen consumption of 9.7 ml. per 100 ml. blood—assuming complete oxidation of glucose. They found that the average difference between the arterial and cerebral venous blood was 9.3 ml. per cent. Gerard and Schachter (1932) have made similar experiments and calculations.

These observations make it highly probable that glucose oxidation is at any rate quantitatively the most important respiratory process in the brain *in vivo*.

Respiration of brain in vitro. Much information on this topic[3] is now available and reference may be made to existing reviews of the subject (Page, 1937; Winterstein, 1932; Gerard, 1937; Holmes, 1935).

Manometric methods of Barcroft and of Warburg have been usually

[3] Gerard (1932) has reviewed the subject of nerve metabolism so far as 1932. Further information, after that time, may be found in the Annual Reviews of Biochemistry.

adopted for the estimation of the amounts of oxygen consumed by brain tissue *in vitro*. Details of these methods will not be described here; they may be found in the publications of the workers in this field. In particular the reader may be referred to the papers of Warburg (1930) and his colleagues in which the history of manometric methods, the technical details and the conditions under which such methods should be adopted are given in detail. In addition the reader may be referred to a publication of M. Dixon (1934).

Brain tissue is usually examined in a minced or chopped condition or a tissue brei, and in the form of thin slices usually of cerebral cortex. The tissue is suspended in a suitable medium and measurements of oxygen uptake are made. Oxygen instead of air is present in the manometric vessels when slices are used and the slices must be of suitable dimensions (0.2–0.4 mm. thick) to ensure adequate diffusion of oxygen and not so fragile that they are distintegrated at the termination of the experiment.

A number of workers have considered that so far as the oxidative mechanisms of brain are concerned the process of chopping has little deleterious action. Thus Ashford and Holmes (1931) compared the oxygen uptake of intact freshly dissected frog's brain with that of chopped frog's brain and found that the total oxygen uptake of the chopped brain in four hours at 19° was 16 per cent less than that of the intact tissue. This suggests that chopping brain brings about little effect on the respiration. According to M. Dixon and Elliott (1929) the rates of respiration of sliced and minced tissues are the same and it is assumed that the process of slicing according to Warburg's technique (1923)—with a razor moistened with Ringer solution—does the least possible damage to the tissue. Holmes (1930) thinks that so far as the oxygen uptake of the brain cortex is concerned "it matters little whether it is chopped or sliced." This view is supported to some extent by the evidence of Quastel and Wheatley (1932) who find that when chopped guinea-pig brain is allowed to take up oxygen for two or three hours and the rate of oxidation has fallen considerably owing to depletion of metabolites, the addition of glucose restores the rate of oxidation to a value not remotely removed from the initial level. Had the processes of chopping and shaking the tissue brei in a glucose free phosphate saline medium for 2 or 3 hours at 37° brought about a serious disturbance in the oxidative mechanism, the observed restoration of oxygen uptake on addition of glucose would not have been expected. It is abundantly clear in the case of another organ, liver, that the process of chopping

very greatly affects oxidative systems—for in this case the oxidation of fatty acids, which takes place at a rapid rate with liver slices, is almost completely absent in a liver *brei* (Quastel and Wheatley, 1933).

Thus the evidence points to a comparative stability of oxidative mechanisms in brain or to a comparatively high resistance of brain cells to mechanical injury.[4] It is clear, however, that the process of mincing does involve changes which should be considered in any quantitative work with brain. For example, when minced brain, which has respired in a glucose medium, is centrifuged and the centrifuged deposit placed in a fresh glucose medium, there is a drop of 40 per cent in the rate of respiration. When brain slices, on the other hand, which have also respired in a glucose medium, are removed from this medium, washed in a Ringer medium and placed in a fresh glucose medium, the subsequent rate of respiration does not show a fall in respiration greater than 8 per cent (Quastel and Wheatley, 1934). It would appear that a factor, essential for glucose oxidation in brain, diffuses more easily from chopped brain than from brain slices.

T. F. Dixon and Meyer (1936) working with ox brain have found that the respiration of minced cerebral cortex in presence of glucose is about half that of the sliced tissue. The respiration of other parts of the brain (cornu ammonis, globus pallidus, thalamus, cerebellar cortex) in presence of glucose is very greatly decreased, on mincing, from the values obtained with intact slices. Clearly various parts of the central nervous system have different susceptibilities to mechanical injury so far as total respiration is concerned. This seems to be particularly the case with the larger cells of the gasserian ganglion. T. F. Dixon and Meyer point out that this fact invalidates the conclusion of Holmes (1932) who, using chopped tissue, found that the respiration of the gasserian ganglion is very low in a glucose medium, this being actually less than that of the cranial nerve. Holmes had suggested on the basis of this result that the seat of metabolic activity may be in the dendrite rather than in the cell body, since the gasserian ganglion cells have no dendritic structures.

It is quite conceivable that the failure of other workers to note large differences between the respirations of sliced and minced tissues has been due to their working with the whole brains of small animals; possibly less mechanical injury on the brain as a whole is effected in

[4] It should be pointed out that, according to Cohen and Gerard (1937), a colloidal aqueous extract of brain can still accomplish various oxidations, but not that of glucose.

these cases. Weil-Malherbe (1937) has recently drawn attention to the fact that the extent of succinic acid formation, during pyruvic acid metabolism, depends greatly on whether sliced or minced tissue is used, and Jowett (1938) has shown that the respiration of sliced brain is more sensitive to a narcotic such as ethyl urethane than was found by Bülow and Holmes (1932) who used minced brain.

TABLE 2

BRAIN CORTEX SLICES	Q_{O_2}	MEDIUM	REFERENCE
Rat	10.7	Ringer-glucose	Warburg (1924)
Rat	14.5	Ringer-glucose	Loebel (1925)
Rat	12.4 (13.7–11.4)	Ringer-glucose	Dickens and Šimer (1930 a, b)
Rat	19.1*	Phosphate-saline-glucose	Jowett and Quastel (1937 c)
Guinea pig	14–15	Phosphate-saline-glucose	Quastel and Wheatley (1934)
Guinea pig	11.8 (over 2 hr. period)	Phosphate-saline-glucose	Jowett and Quastel (1937 b)
Guinea pig	2.7	Ringer only	Wohlgemuth (1933)
Rabbit	6.0	Ringer only	Holmes (1930)
Rabbit	7.3–10.4	Rabbit serum	Krebs and Rosenhagen (1931)
Rabbit	8.3	Ringer-glucose	Dixon (1936)
MINCED BRAIN TISSUE			
Rat	5.3	Phosphate-saline	Quastel and Wheatley (1932)
Guinea pig	3.8		Szent-Gyorgyi (1931)
Rabbit	3.8	Bicarbonate-Ringer	Ashford and Holmes (1931)
Pigeon	3.3	Phosphate-Ringer	Peters and Sinclair (1933)
Guinea pig	3.3–4.6	Phosphate-saline	Quastel and Wheatley (1932)
Guinea pig	6.7	Phosphate-saline-glucose	Quastel and Wheatley (1934)

* Estimated over the first 24 minutes of the experiment. The respiration of rat brain cortex slices falls with time.

Rate of oxygen uptake by brain tissue. Typical results showing the magnitude of the respiration of isolated brain tissue are given in table 2.

It will be noted *a*, that the Q_{O_2} of brain cortex slices is decidedly higher in a glucose medium than in a glucose free medium.

b, that the Q_{O_2} of minced whole brain is lower than that of cortex

slices. This is partly due to the fact that whole brain contains white matter which respires at a lower rate than gray matter. Results with minced whole brain, in absence of added glucose, are also variable because the amount of glucose in the brain when removed from the animal is a variable quantity.

T. F. Dixon and Meyer (1936) have examined the respirations of slices of various parts of ox brain in presence of glucose and give the following results:

	O_2 uptake in µl. per gram wet weight per hour
Cerebral cortex	1,700
Cerebellar cortex	2,550
Corpus striatum	1,980
Cornu ammonis	1,260
Thalamus	1,170
Globus pallidus	360

There is little doubt that the Q_{O_2} of brain cortex slices examined under the most favorable conditions so far is lower than that expected from the values of oxygen consumption of brain *in vivo*. Conceivably the latter are too high, but it is more likely that the optimal conditions for the examination of brain tissue *in vitro* have not yet been secured. The respiration of brain cortex slices in a glucose medium varies with time; it falls off much more with time in the case of rat than in the case of guinea pig, and the rate of fall depends very greatly on the ionic concentration of the medium. With rat cortex a Q_{O_2} of 19.1 was found during the first 24 minutes of the experiment; a higher figure would obviously have been obtained had it been possible to make an accurate estimation during the first few minutes of examination of the tissue. Even this figure is not remote from the values calculated from the *in vivo* studies.

Brookens, Ectors and Gerard (1936) have examined the respiration of plugs of cerebral cortex with a capillary respirometer. They have found values of $Q_{O_2} = 10$, but with a technique permitting readings within the first few minutes a linear extrapolation to zero time gave values of $Q_{O_2} = 25$.

Respiratory quotient of brain in vitro. Loebel (1925) showed that the R. Q. of brain slices in a fructose medium was 0.99. Dickens and Šimer (1930b, 1931) found that the R. Q. of normal rat brain cortex slices in a glucose medium is 0.99.

Wortis (1935) investigated the respiratory activity of minced brain tissue of cat and rat and found under normal conditions an R. Q. of unity. Himwich and Fazekas (1932) have determined the respiratory quotients for various parts of rat brain and have obtained the following results: cortex, 0.99; brain stem, 0.93; cerebellum, 0.89; medulla, 0.89. Dickens and Greville (1933) working with rat and rabbit brain cortex slices obtained an R. Q. of unity which was maintained even in the absence of added glucose to the medium. Elliott and Baker (1935) reported a lower value for the R. Q. of rat brain cortex (0.93–0.78) but Dickens (1936) points out possible inaccuracies in their experiments, and shows that the R. Q. of rat brain cortex in glucose, fructose or lactate media is unity.

It is evident that the respiratory quotient of normal brain examined *in vitro* is approximately unity, a value which agrees with the determinations on brain *in vivo*. Presumably carbohydrate oxidation is responsible for this quotient. This conclusion is supported by the work of K. C. Dixon (1936). This worker showed that the amount of glucose disappearing aerobically in the presence of brain slices is equal to that expected from the oxygen consumption (assuming complete oxidation) and from the amounts of lactic acid formed by the aerobic glycolysis. His figures, using rabbit brain cortex, are as follows:

Q_{O_2} = μl O_2 absorbed per mgm. dry wt. tissue per hr. = 8.3

$\therefore$ Glucose oxidised to account for this consumption of O_2, $= \frac{8.3}{6}\mu l = 1.38\,\mu l$ since 1 mol. glucose requires 6 mols O_2 for complete oxidation.

$Q_M^{O_2}$ = aerobic glycolysis = μl lactic acid formed aerobically per mgm. dry wt. tissue per hr. = 2.8 μl

$\therefore$ Glucose broken down to account for this amount of glycolysis $= \frac{2.8}{2} =$ 1.4 μl since 1 mol. glucose produces 2 mols lactic acid.

$\therefore$ Total glucose broken down = 1.38 + 1.4 = 2.78 μl per mgm. dry wt. tissue per hour.

Amount of glucose found to be broken down aerobically = 2.4 μl; 2.6 μl; 3.5 μl; 3.2 μl; 4.0 μl (average = 3.14 μl) per mgm. dry wt. tissue per hour.

The correspondence is reasonably good, and indicates that the respiration of brain cortex *in vitro* can be mainly accounted for on the basis of oxidation of glucose.

Effects of change of temperature on brain respiration. K. C. Dixon

(1936) has studied the effects of abnormally high temperatures on the metabolism of cerebral cortex. The following results may be quoted:

	Q_{O_2}			
37°	6.8,	8.7,	8.1,	9.0
42°	8.0,	8.2,	9.4	
45°	17.0,	13.0,	16.8,	16.8.

These results were obtained using rabbit brain cortex in a bicarbonate-Ringer solution containing glucose. At 42° the rate of respiration was only slightly increased above that at 37°, but at 45° the respiration was increased very greatly above that at 37°. Similarly the amount of glucose broken down is greatly increased at 45° over that at 37°.

Dixon cites the following results for the change in respiration and aerobic glycolysis for the temperature range 42–45°.

TEMPERATURE	Q_{O_2}	$Q_M^{O_2}$ (AEROBIC GLYCOLYSIS)
°C.		
42	8.4	2.7
43	10.8	3.4
44	11.3	5.5
45	11.0	11.3

It seems to be clear that above 42° there is a definite change in the equilibria governing glucose breakdown in the brain.

Comparison of respirations of gray and of white matter. The respiratory activity of white matter is about one-third to one-quarter that of of gray matter (cf. Winterstein, 1922). Holmes (1930) gives 1200 μl per gram per hour for gray matter of rabbit brain and 300 μl (Q_{O_2} = 1.5) per gram per hour for the white matter.

Brookens *et al.* (1936) using a capillary micro-respirometer state that respiration values for various portions of white matter range from 650 to 1800 μl O_2 per gram per hour and for gray matter from 800–2500 μl per gram per hour.

Krebs and Rosenhagen (1931) give a range of 2.8–4.6 as the Q_{O_2} for rabbit brain white matter while the Q_{O_2} of gray matter of basal ganglia is given as 7.6–9.9.

Effects of diffusible metabolites on brain respiration. Washed brain tissue (in absence of added metabolites) respires less than fresh unwashed tissue. Holmes (1930) showed that the respiration of mouse brain falls very greatly after the tissue is washed. Peters and Sinclair

(1933) found that the respiration of pigeon brain fell rapidly when the minced tissue was immersed in a Ringer-phosphate medium. They thought this was due to a gradual inactivation of an essential enzyme system, until further study made it evident that the addition of various metabolites (lactate, pyrophosphate and α-glycerophosphate) had a considerable influence on the respiratory rate. Quastel and Wheatley (1932) found that fresh minced guinea pig brain tissue takes up oxygen at a rate of 200 μl per gram per 15 minutes ($Q_{O_2} = 4$) for about two hours after which the respiratory rate begins appreciably to fall reaching about half the initial rate after three hours. On addition of glucose or sodium lactate to the tissue at this stage the rate of oxygen uptake immediately increases. Moreover the addition of glucose to the brain tissue at the commencement of the experiment leads to a much longer maintenance of the Q_{O_2} at the initial level.

There can be little doubt that diffusible metabolites are largely responsible for the maintenance of respiration in brain. The rapid fall in respiration which brain tissue exhibits, in a metabolite free medium, is due partly to depletion of metabolites in the tissue, either by oxidation or by diffusion into the external medium so that their effective concentrations are lowered.

Action of cyanide on brain respiration. Cyanide has a large inhibitory action on brain respiration, but according to M. Dixon and Elliott (1929) and to Banga, Schneider and Szent-Gyorgyi (1931) an appreciable percentage of the respiration is not inhibited by cyanide. It must be borne in mind however that cyanide inhibition depends very much on the experimental conditions (Alt, 1930). Torrès (1935) finds that 97.5 per cent of the respiration of rat brain examined in Ringer solution is inhibited by $\frac{N}{200}$ cyanide; the Q_{O_2} in presence of cyanide being 0.27. According to Himwich *et al.* (1933) cyanide while diminishing the oxygen uptake of brain cortex does not affect the respiratory quotient.

The pathological changes which take place in brain after the administration of cyanide to animals have been investigated by Meyer (1933).

Marshall and Rosenfeld (1937) have studied the action of small doses of cyanide as a respiratory stimulant and have shown that respiratory stimulation from the cyanide may occur in dogs and cats with no decrease in the oxygen consumption of the whole animal. They point out that stimulation from cyanide resembles very closely that from anoxemia and explain this "by assuming that the sino-aortic receptors

are more readily affected than other tissues." It is well known that different tissues vary in their sensitivity to cyanide *in vitro* (cf. Kisch, 1933).

Chang and Gerard (1931) have shown that cyanide largely abolishes the resting oxygen consumption of vertebrate and invertebrate nerve and that the cyanide inhibition can be reversed by the addition of a reducible dyestuff such as cresyl blue.

The large inhibitory effect of cyanide on brain respiration points to the probability that the respiration proceeds largely through the cytochrome-cytochrome oxidase system. The small cyanide-insensitive part of the respiration may be due to the activity of a flavine system.

Action of carbon monoxide. Little is known of the action of CO on brain oxidation but Chang and Gerard (1931) have recorded its effects on nerve respiration. In 98 per cent CO + 2 per cent O_2 the resting respiration of frog nerve, in presence of cresyl blue, is depressed 70 per cent in the dark and less than 5 per cent in the light. Schmitt (1930) discusses the inhibition of nerve respiration by CO and the action of the light. The action of CO on the metabolism of retina, among other tissues, is described by Laser (1937).

The fact that intact nerve in presence of CO does not respond to the action of a dye to the extent that nerve in presence of cyanide responds makes it likely that the actions of CO and of cyanide on nerve respiration are not identical. The reader is referred, however, to Gerard's monograph (1932) for details concerning nerve metabolism.

The pathological changes found in brain following carbon monoxide have been described by Meyer (1932).

Indophenol (cytochrome) oxidase of brain. Indophenol oxidase activity of brain was studied by Vernon (1911) who showed that the intensity of the reaction varied inversely with the size of the animal. The addition of *p*-phenylenediamine to brain tissue greatly increases its oxygen uptake. Holmes (1930) finds that the ratio of oxygen uptakes in presence of *p*-phenylenediamine with various parts of cat brain is as follows: gray matter: white matter: nerve = 26.2:4.3:1. Quastel and Wheatley (1932) have examined the activities of brain tissue of various animals towards *p*-phenylenediamine and their results are shown in the accompanying table (table 3) taken from their paper.

Fresh brain tissue was used in the experiments referred to in table 3 (with the exception of man). The relatively high value of the autoxidation of these tissues makes it difficult to estimate precisely the amount of oxidation of *p*-phenylenediamine, for there may be competition be-

tween the diamine and the substrates responsible for the autoxidation. Clearer results were obtained with tissues which had been allowed to deplete themselves considerably of their substrates by respiration in a

TABLE 3

O_2 uptake in μl. (at 37°) by 0.5 gram minced whole brain tissue alone and in the presence of 21 mgm. p-phenylenediamine hydrochloride (neutralised) in 90 minutes

Theoretical O_2 uptake for combination of one atom O_2 with this quantity of p-phenylene diamine =1474 μl.

	ALONE	WITH *p*-PHENYLENEDIAMINE	EXTRA O_2 UPTAKE
Mouse	696	2,212	1,516
Rat	680	1,428	748
Guinea pig	645	1,480	835
Rabbit	685	1,525	840
Pigeon	844	2,310	1,466
Cat (grey matter)	422	1,560	1,138
Man (grey matter, from brain twelve hours after death)	444	1,402	958

phosphate medium for 3 hours at 37° before the addition of the diamine. The following results were then obtained (conditions as in table 3):

	ALONE	O_2 UPTAKE μL. IN 1 HOUR WITH *p*-PHENYLENEDIAMINE	EXTRA O_2 UPTAKE
Rabbit	162	1315	1153
Mouse	154	1534	1380
Guinea pig	154	1406	1252

The rates of oxygen uptake in presence of *p*-phenylenediamine (i.e., the relative indophenol (or cytochrome) oxidase activities) were inversely proportional to the size of the animal. Considering the wide difference between the indophenol oxidase activities of gray and of white matter, it is possible that the inverse relationship noted is due to varying proportions of gray to white matter in the brains of these animals, the smaller animals having the greater proportion of gray matter.

Quastel and Wheatley (1932) found the Q_{O_2} of brain in presence of *p*-phenylenediamine to rise rapidly from a value of 4 to a value of about 17 and then to fall rapidly as the diamine became exhausted within the next 90 minutes to a Q_{O_2} of about 2. There is evidence that the products

of oxidation of the diamine inhibit the oxidation by brain of its normal substrates.

These facts make it clear that brain in common with other mammalian tissues possesses an enzyme for the activation of molecular oxygen. The strong inhibitive action of cyanide on normal respiration of brain points to the fact that the indophenol (cytochrome) oxidase is quantitatively the most important mechanism for oxygen activation.

So far as other cyanide sensitive enzymes are concerned, peroxidase exists to a small extent in brain and so does catalase, though the amount of the latter is slight, perhaps one hundredth of the amount in liver (Schmitt and Skow, 1933; Elliott and Bancroft, 1934).

TABLE 4

Q_{O_2} of minced guinea-pig brain which had been allowed to respire for 3 hours in phosphate saline medium prior to the addition of sugars

The initial Q_{O_2} was 4.0

SUGAR (0.05 PER CENT)	TIME FROM ADDITION OF THE SUGAR	
	30 minutes	120 minutes
None (control)	1.7	1.1
Glucose	2.9	4.0
Fructose	3.5	3.5
Mannose (0.025%)	2.8	2.9
Galactose	2.5	1.7
Mannitol	1.9	1.2
Xylose	1.5	1.3
Arabinose	1.3	1.0
Glycogen	1.8	1.7

Cytochrome. Cytochrome exists in brain but little is known about its distribution in this organ. According to Holmes (1932) there is about half as much cytochrome in cerebral cortex as in yeast, more than in skeletal muscle but less than in heart muscle. White matter from the cord contains one-quarter to one-fifth as much as cortex and it cannot be stated with certainty whether any cytochrome exists in nerves.

Oxidation of carbohydrates by brain in vitro. In confirmation of the known facts concerning R. Q. of brain and glucose removed from the blood in its passage through the brain, the addition of glucose to brain tissue *in vitro* greatly prolongs the steady rate of oxygen uptake. Fructose and mannose also increase the oxygen uptake of brain tissue (Loebel.

1925). The work of Dickens and Simer (1931), Ashford and Holmes (1931), Quastel and Wheatley (1932) indicates the importance of glucose oxidation to brain *in vitro*. The last workers studied the effects of various sugars on the oxidations by brain tissue which had been allowed to deplete itself largely of oxidizable material, prior to the addition of the sugar, by autoxidation for three hours. Typical results are shown in table 4. Of all the sugars tested, glucose, fructose and mannose have the largest influence in restoring the Q_{O_2} to a rate approaching the initial value. Galactose has a small effect, but mannitol, arabinose and xylose are practically inert at the concentrations tried (see also Sherif and Holmes, 1930).

The relative rates of oxidation of glucose by minced brain tissues from various animals are shown by the following figures: (the brain tissue had been allowed to respire for 3 hours prior to the addition of the glucose). The figures refer to μl O_2 uptake in 1 hour at 37° by 0.5 gram brain tissue.

ANIMAL	NO GLUCOSE ADDED	GLUCOSE (0.025 PER CENT) ADDED
Rabbit	162	229
Guinea pig	154	287
Mouse	154	345

It will be observed that, as in the case of indophenol oxidase activity, the smaller the animal the greater the rate of oxidation of glucose by the brain.

With minced pigeon brain (cerebrum) Gavrilescu *et al.* (1932) obtained the following figures.

MEDIUM	O_2 UPTAKE μL. PER GRAM PER HOUR
Ringer	775; 805
Ringer + glucose 0.25 per cent	1645; 1955

The respiration of brain cortex slices falls quickly with time at 37° in a phosphate-Ringer medium in the absence of glucose. With glucose present the rate remains approximately constant for at least two hours. The values given in table 5 illustrate this fact; they refer to guinea-pig brain cortex.

TABLE 5

MEDIUM	Q_{O_2} TIME FROM COMMENCEMENT OF THE EXPERIMENT				REFERENCE
	30 minutes	60 minutes	90 minutes	120 minutes	
Phosphate-glucose 0.16%.....	15.8	16.2	16.7	14.3	Quastel and Wheatley (1934)
Bicarbonate-glucose 0.2%.....		10.4		9.1	Dickens (1936)

With rat brain cortex in a phosphate medium the Q_{O_2} falls more rapidly than with guinea-pig brain. Typical values (Jowett and Quastel, 1937) for the respiration of rat brain cortex in the absence and in the presence of glucose and in a phosphate-saline medium are as follows:

Q_{O_2} (mean value for a 2 hour period)

No glucose added.....	2.89 ± 0.18
With glucose 0.01 M.....	12.20 ± 0.33

Dickens and Greville (1933) give the following figures as illustrative of the respiration of rabbit and rat brain cortex in Ringer media with and without carbohydrate

	Q_{O_2}	
	Rat	Rabbit
No carbohydrate added.....	3.9	5.0
Glucose present.....	12.8	7.5
Fructose present.....	9.7	8.5

Holmes (1932) showed that the respiration of the brain of an animal killed after it had been allowed to convulse after administration of insulin was less than that of a normal animal—the brain tissues being examined in glucose free media. This is due to the fact that the respiration of brain in a glucose-free medium depends upon its store of metabolites, of which glucose (or lactic acid) is the most important. The amount of this in the brain is lessened when the blood sugar falls after insulin administration. Wortis (1935) confirmed this fact, using minced brain, and gave the figures shown in table 5a (see also Wortis, 1938).

His results indicate that the brain of an insulin treated animal respires normally in a glucose medium;[5] the fall in respiratory activity as well as the drop in R. Q. in a glucose-free medium shows that the brain of such an animal must contain very little glucose. These observations are also confirmed by the work of Himwich and Fazekas (1937), who have shown that the difference between the glucose contents of arterial blood and cerebral venous blood of dogs under intense hypoglycemia is only 3 mgm. per cent, as compared with a normal difference of about 13 mgm. per cent. Moreover the oxygen consumption of dog brain *in vivo* falls from a normal figure of 7.99 volumes per cent to 3.80 volumes per cent during intense hypoglycemia.

TABLE 5a

	R.Q.		Q_{O_2} (60 minutes)	
	Ringer-phosphate	Ringer-phosphate-glucose 0.2%	Ringer-phosphate	Ringer-phosphate-glucose 0.2%
Normal rat brain	1.00	1.01	3.6	5.5
Brain of rat injected with insulin until convulsions occurred	0.73	0.92	1.4	5.2

There can be little doubt that during the hypoglycemia induced by insulin there is a lessened consumption of oxygen by brain and lessened glucose utilization by this organ. The intravenous injection of glucose to a hypoglycemic animal restores the oxygen consumption by the brain *in vivo* to the normal level.

Dameshek, Myerson and Stephenson (1935) compared the contents of glucose and of oxygen in the vessels supplying the human brain before and after intravenous injection of insulin. They took samples of blood from the brachial artery and the internal jugular vein and came to the conclusions that the uptake of glucose by the brain is materially reduced during a severe hypoglycemic reaction and that the uptake of oxygen by the brain varies indirectly with the severity of the insulin reaction, becoming much reduced during the most severe reactions.

Himwich, Bowman, Wortis and Fazekas (1938b) have confirmed these findings. They have examined the oxygen content of blood from the internal jugular vein and femoral artery in schizophrenic patients and the same patients treated with insulin, in the hypoglycemic "shock"

[5] See also Gerard and Schachter (1932).

therapy. They state that before insulin is injected the average oxygen utilization is 7.04 vols. per cent, and that of glucose 12.5 mgm. per cent. After insulin injection, whilst the patients are in the pre-comatose condition, the oxygen consumption falls to 6.1 vols. per cent and the utilization of glucose falls to 7 mgm. per cent. It is evident that insulin, in lowering the blood glucose, diminishes access to the entire central nervous system of its most important substrate for respiratory purposes.[6] Probably the various neurological disturbances which manifest themselves during hypoglycemia are due to the resulting anoxemia in the nervous system although oxygen is freely available as shown by the relatively high oxygen content of the cerebral venous blood.

The brain tissue of depancreatized or diabetic animals burns lactic acid and glucose (Holmes and Holmes, 1927; Baker, Fazekas and Himwich, 1938) at the normal rates. Table 6 shows results given by the latter workers.

TABLE 6

CEREBRAL CORTEX OF	GLUCOSE (0.02M)		LACTATE (0.02M)	
	Q_{O_2}	R.Q.	Q_{O_2}	R.Q.
Normal cat	9.1	0.95	7.8	1.01
	8.7	0.94	10.7	0.88
	7.1	0.99	8.7	0.97
Diabetic cat	8.8	0.97	9.7	0.86
	8.9	1.01	9.1	0.90
	10.2	0.98	9.6	1.03

It is evident that insulin lack does not affect the ability of brain to burn glucose. The addition of insulin to a glucose-Ringer medium has no effect on the glucose oxidation by brain (Wortis, 1938).

Carbohydrate breakdown in brain. Glucose is not converted into glycogen by brain (Holmes and Ashford, 1930) and apparently glycogen[7] is not stored by brain (Takahashi and Asher, 1925). It is evident, however, from the results of Kerr and Ghantus (1936) and Kerr (1938) that glycogen is definitely present in the brain. The results of Gaddie and Stewart (1935) and Geiger (1935) make it clear that the glycogen breakdown in brain is small compared with that in muscle.

[6] Kerr, Hampel and Ghantus (1937), and Kerr and Ghantus (1936), have pointed out that insulin administration decreases the levels of both glycogen and free sugar in the brain of normal cats.

[7] For a short review of glycogen metabolism in brain see Page, *Chemistry of the Brain*, 1938, p. 183.

Holmes (1930) and Krebs (1931) have given reasons for the belief that glucose oxidation in brain proceeds through lactic acid. This conclusion, however, can no longer be held owing to the recent finding that lactate oxidation in presence of brain can be inhibited to a greater extent than glucose oxidation. This finding was made independently by Jowett and Quastel (1937a), and by Baker, Fazekas and Himwich (1938). (See also Himwich and Fazekas, 1935.) The former used as

TABLE 7

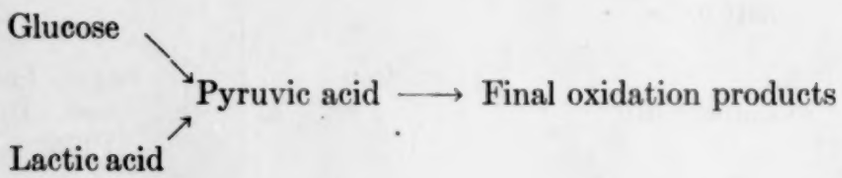
ANIMAL	INHIBITOR	Q_{O_2} SUBSTRATE		PER CENT EFFECT OF INHIBITOR ON Q_{O_2}		REFERENCE
		Glucose 0.02M	*dl* lactate 0.02M	Glucose	*dl* lactate	
Guinea pig	Nil	15.8	11.1*			Jowett and Quastel (1937 a)
	Sodium hydroxy-malonate 0.07M	11.1	6.6*	−30	−52	
Rat	Nil	13.8	13.1*			Jowett and Quastel (1937 a)
	Sodium hydroxy-malonate 0.07M	12.5	9.2*	−9	−30	
Rat	Nil	15.0	15.0			Baker, Fazekas and Himwich (1938)
	Nicotine 0.03M	8.3	1.8	−45	−88	
Cat	Nil	9.0	9.4			Baker, Fazekas and Himwich (1938)
	Nicotine 0.03M	6.5	1.3	−28	−86	
Rat	Nil	13.5	13.5			Baker, Fazekas and Himwich (1938)
	Nicotinic acid 0.03M	12.5	4.2	−7	−69	

* = Lactate 0.01M

inhibitor sodium hydroxymalonate which is known to be a specific inhibitor of lactic dehydrogenase (Quastel and Wooldridge, 1928); the latter used nicotine whose mechanism of action on enzymic reactions is as yet unknown. Relevant figures showing the actions of these inhibitors on lactate and glucose oxidation by brain cortex are given in table 7.

Glucose cannot be directly oxidized to CO_2 and water, i.e., with-

out the formation of intermediate substances, for the addition of glucose to brain tissue already burning lactate does not increase the rate of oxygen uptake (Quastel and Wheatley, 1932), a fact confirmed by Elliott, Greig and Benoy (1937). It was thought originally that this supported the contention that glucose oxidation proceeds *via* lactic acid, but in view of what has already been stated, another explanation must be sought for the lack of additive action. Such an additive action should take place if the oxidations of glucose and of lactic acid were quite independent. It is improbable that lactate oxidation proceeds through glucose as an intermediary because *a*, concentrations of fluoride and iodoacetate which inhibit glucose oxidation do not inhibit lactate oxidation (Holmes, 1930; Krebs, 1931; Quastel and Wheatley, 1932); *b*, brain slices fail to synthesize glucose from lactate under conditions where synthesis takes place with liver and kidney slices (Benoy and Elliott 1937). The most obvious explanation of the fact that glucose and lactate oxidations show no additive effects is that both substances proceed through a common intermediate, whose oxidation accounts for most of the oxygen uptake. Such an intermediate might be pyruvic acid, thus:



According to Elliott *et al.* (1937) the addition of glucose does not increase the rate of respiration of rat brain cortex in presence of pyruvate.

It has long been known that fructose can be oxidized by brain at a rate comparable with that of glucose, although little breakdown of fructose to lactic acid takes place in presence of brain (Loebel 1925). Haarman (1932) gives the ratio of glycolytic activities of glucose, fructose, hexosediphosphate and glycogen in presence of brain tissue (rabbit, dog) as 25.4: 3.7: 3.2: 1.0. Dickens and Greville (1933) conclude from their experiments that fructose is directly oxidized by brain cortex. Iodoacetate inhibits fructose oxidation by brain (Quastel and Wheatley, 1932) and it is conceivable that fructose is oxidized by a path similar to that of glucose.

Oxidation of lactic acid in the brain. Lactic acid arises from glucose in presence of brain, both aerobically and anaerobically, the rate of anaerobic formation being far greater than that aerobically. A dis-

cussion, however, of glycolysis in brain and of the action thereon of oxygen, is outside the scope of this review.

Lactic acid is a normal constituent of blood and must be considered, therefore, as a factor contributing to the respiration of brain: Himwich *et al.* (1938) state that it is doubtful whether lactic acid is utilized by the brain to any appreciable extent *in vivo* and give figures which make it seem unlikely that lactic acid is removed from the blood on its passage through the human brain. It seems, however, that their experiments are by no means conclusive on this point, and the question of whether lactic acid is oxidized by brain *in vivo* must be left in abeyance until further work has been done. Lactic acid, like glucose, is stated to be absorbed from the blood by the brain of normal dogs (McGinty, 1929) and by the brain of diabetic dogs (Himwich and Nahum, 1929).

TABLE 8

PER CENT CONCENTRATION OF LITHIUM LACTATE ADDED	μL O_2 EQUIVALENT TO LACTIC ACID DISAPPEARANCE	TOTAL μL O_2 TAKEN UP	RATIO
0.025	2350	4710	0.5
0.05	3000	4875	0.61
0.1	4450	5000	0.89
0.2	6750	6450	1.05
0.3	9200	6700	1.37
0.4	8300	6480	1.28
0.5	8260	6300	1.30
0.6	10000	7100	1.40
0.8	10500	7000	1.44

There is no doubt that lactic acid prolongs the oxygen uptake of brain (Loebel, 1925; Meyerhof and Lohmann, 1926; Ashford and Holmes, 1929). The last authors made a careful examination of the disappearance of lactic acid acid aerobically in brain and have shown that more lactic acid disappears than can be accounted for by the extra oxygen uptake due to the added lactate. No satisfactory explanation of this phenomenon is as yet forthcoming. An obvious explanation is that the lactic acid exercises a "sparing" action on the oxidation of other metabolites, i.e., that the "extra O_2" is not a true indication of the amount of lactic acid oxidized. Moreover such a sparing action can be experimentally demonstrated. But in certain of the experiments of Ashford and Holmes the *total* oxygen uptake of the brain is less than that expected for the complete oxidation of the lactic acid which has disappeared. Table 8, taken from their paper, illustrates this.

It is evident from these data that with concentrations of lithium lactate above 0.2 per cent, the discrepancy between O_2 uptake and the lactic acid removed cannot be explained entirely by the sparing action of lactate. At the lower concentrations, however, which are more biologically significant, a sparing effect would be an ample explanation.

Quastel and Wheatley (1932) have shown that the addition of lactate and pyruvate to brain tissue, partially depleted of its metabolites by autoxidation for 3 hours, restores the rate of respiration practically to that found with the fresh tissue. The rate of oxidation secured by lactate (*dl*) is almost identical with that obtained by an equivalent concentration of glucose; this fact affords additional evidence for the view that the oxidations of glucose and of lactic acid proceed through the intermediate formation of a common step—possibly pyruvic acid. These authors also showed that the presence of *dl* lactate exerts a "sparing" effect on the oxidation of sodium succinate by brain tissue.

Peters and Sinclair (1933) observed that pyrophosphate increases the rate of oxidation of lactate by minced pigeon brain; the effect is enhanced by the addition of magnesium ions. According to these authors one-third of the respiration of pigeon brain tissue in presence of lactate is inhibited by fluoride. They found that the addition of pyruvate to lactate caused a slight increase in respiration of minced pigeon brain.

The fact that biological oxidation of lactic acid leads to the formation of pyruvic acid is well known. The rapid rate of pyruvic acid oxidation by brain tissue has made it difficult to detect pyruvic acid formation when lactic acid is being burned by brain. That it is formed under such circumstances, however, has been shown in the case of minced pigeon brain by Peters and Thompson (1934) and in the case of rat and guinea-pig brain cortex slices by Jowett and Quastel (1937b). The amounts of pyruvic acid found are, as might be expected, small; the concentration found in a lactate medium in which brain tissue is respiring is presumably in dynamic equilibrium with that in the tissue slices. The pyruvate concentration found with guinea-pig or rat brain cortex slices in a 0.02 M *d* lactate medium is of the order 2×10^{-4} M.

The amount of pyruvate found in a lactate medium in which pigeon brain tissue is respiring is, according to Peters and Thompson (1934) markedly increased, if iodoacetate (0.0005 M) is also added to the medium. Presumably the iodoacetate inhibits the breakdown of pyruvate. Further work showing that lactic acid oxidation in pigeon brain proceeds through pyruvic acid has been carried out by Peters (1936).

As has already been pointed out hydroxymalonate which is an inhibitor of lactic dehydrogenase inhibits the oxidation of lactic acid by brain (table 7). Lactic dehydrogenase, as well as other dehydrogenases, exists in brain tissue, their presence having been demonstrated by the anaerobic methylene blue technique.

Herter in 1905 showed that methylene blue is reduced by brain *in vivo*, the reduced dye being reoxidized on exposure to air. Thunberg and Sherif have shown nerve tissue to possess dehydrogenase systems.

Holmes (1930) using the methylene blue technique gives the figures shown in table 9 illustrating the activity of ox brain, and pointing clearly

TABLE 9

	REDUCTION TIME	
	Grey matter	White matter
	minutes	*minutes*
Control (no added substrate)	13½	70
M/50 Lithium lactate	7	24
M/100 Glucose	6	48
M/100 Galactose	10	70

TABLE 10

SUBSTRATE	M CONCENTRATION AT WHICH HALF THE MAXIMUM VELOCITY OF REDUCTION IS REACHED
Glucose	0.0135
Fructose	0.021
Sodium lactate	0.0045
Sodium pyruvate	0.0052
Sodium citrate	0.018
Sodium succinate	0.004

to the existence of lactic dehydrogenase in brain. The amount of the dehydrogenase is greater in gray than in white matter.

Ashford and Holmes (1931) state that lactate and glucose have approximately the same activities as hydrogen donators to methylene blue. Davies and Quastel (1932) find, however, differences between these activities as shown in table 10 taken from their paper. This table gives the Michaelis constants of various hydrogen donators in presence of minced ox brain tissue.

A study of the lactic dehydrogenase system of brain and of other

organs has been carried out by Green and Brosteaux (1936). It is now well known that a necessary part of the lactic dehydrogenase system in mammalian tissue is cozymase and it has been shown recently by Quastel and Wheatley (1938) using their ferricyanide technique, that the presence of cozymase markedly increases the oxidation of *dl* lactate (by ferricyanide) in rat brain cortex slices. Since the system lactate-lactic dehydrogenase-cozymase reduces ferricyanide but not methylene blue, and since brain tissue in presence of lactate reduces both ferricyanide and methylene blue, it follows that brain contains a factor which transfers hydrogen from reduced cozymase to methylene blue. Evidence for such a carrier has now been obtained by Euler and Hellstrom (1938) and by Dewan and Green (1938).

That cozymase exists in brain has been shown by Sym, Nilsson and Euler (1930) who give the values shown in table 11.

TABLE 11

	COZYMASE UNITS
Brain grey matter	3.7
Brain white matter	1.0
Kidney cortex	5.2
Liver	3.2
Blood	0.1

Oxidation of pyruvic acid by brain. A number of workers has shown that pyruvate is highly effective in maintaining the respiration of brain, either minced or in the form of slices (e.g. Loebel, 1925; Ashford and Holmes, 1931; Quastel and Wheatley, 1932; Peters 1936; Lipmann 1937; Elliott, Greig and Benoy, 1937). With rat brain cortex there is little difference between the Q_{O_2} in presence of glucose 0.01 M and the Q_{O_2} in presence of pyruvate 0.02 M; if anything the respiration may be a little less in pyruvate. The following figures may be cited as typical (Jowett and Quastel, 1937b).

Substrate	Q_{O_2}
None added	2.89 ± 0.18
Glucose 0.01 M	12.20 ± 0.33
Pyruvate 0.02 M	11.10 ± 0.65

It has been known for some time that pyruvate increases the respiration of muscle, liver and kidney in a similar manner to lactate (Meyerhof 1930; Dickens and Šimer 1930b).

That pyruvic acid disappears when brain tissue respires in presence

of it is also now well known (Peters and Thompson, 1934, etc.). The rate of aerobic disappearance is given in table 12.

The disappearance of pyruvic acid is greater than can be accounted for by the oxygen uptake, assuming that complete oxidation of pyruvic acid takes place. This is seen in the results of Jowett and Quastel (1937b) and of McGowan (1937), the former working with rat brain and the latter with pigeon brain. The inference would be that pyruvic acid undergoes a secondary change in presence of brain tissue. A part of this change consists of reduction to lactic acid. Haarman (1932) showed that brain tissue reduces pyruvate to lactate under anaerobic conditions. Elliott *et al.* (1937) found that in a mixture of glucose and pyruvate, in presence of rat brain cortex examined aerobically, most of the pyruvate disappearance can be accounted for by lactate formation. It is evident that this is not the case in the absence of glucose. Krebs

TABLE 12

BRAIN CORTEX OF	PYRUVATE CONCENTRATION	Q_p (RATE OF DISAPPEARANCE OF PYRUVIC ACID) IN μL PER MGM. DRY WEIGHT TISSUE PER HOUR	REFERENCE
Rat.....	0.04M	4.3; 6.2	Elliott, Greig, and Benoy (1937)
Pigeon.....	0.005M	4.3	Lipmann (1937)
Rat.....	0.01M	7.6; 8.4	Jowett and Quastel (1937 b)
Rat.....		9.3; 8.3	Krebs and Johnson (1937 a)

and Johnson (1937) have shown that anaerobically two molecules of pyruvic acid undergo a Cannizzaro reaction in presence of brain tissue to form acetic, lactic and carbonic acids in equimolecular quantities. This has been confirmed by Lipmann (1937) and Weil-Malherbe (1937a). The same phenomenon was shown earlier by Nelson and Werkman (1936) to take place in the presence of bacteria (*lactobacillus lycopersici*), confirmed by Krebs (1937) for staphylococci and indeed Quastel and Stephenson some years earlier (1925) suggested that this reaction would account for the anaerobic growth of *B. coli* in pyruvate media. Assuming, however, that the Cannizzaro reaction does take place aerobically in brain tissue, McGowan (1937) comes to the conclusion that the lactic acid formation aerobically does not account for that amount of pyruvate disappearance which cannot be accounted for by the oxygen uptake.[8]

[8] Long, 1938, has very recently shown that lactic and acetic acids are formed when minced pigeon brain respires in a pyruvate medium, the quantities accounting for 30 per cent of the pyruvate disappearing. The rest of the pyruvate seems finally to be completely oxidized.

It seems fairly clear that pyruvic acid may undergo a variety of reactions, and at present intensive research is being carried out in various laboratories to discover the nature of these reactions. It has been shown of late (Kritzmann, 1938; see also Braunstein and Kritzmann, 1937) that in brain tissue pyruvic acid reacts with glutamic acid to form alanine, a transference of the amino group taking place. Weil-Malherbe (1936) showed that brain slices synthesize glutamine from pyruvic acid and ammonia, α-ketoglutaric acid being supposed to be the intermediate in this reaction. He found (1937b) that succinic acid is formed when pyruvic acid or α-ketoglutaric acid is added to brain under anaerobic conditions. Aerobically succinic acid is not found with brain slices or minced brain, presumably owing to the activity of the succinic dehydrogenase present; in presence of malonate, an inhibitor of this enzyme, some succinic acid accumulates with minced brain tissue but not with brain slices. The mechanism by which pyruvic acid is transformed

TABLE 13

SUBSTRATE	Q_{O_2} IN ABSENCE OF INHIBITOR	Q_{O_2} IN PRESENCE OF SODIUM HYDROXYMALONATE 0.07M	PER CENT INHIBITION
dl Lactate 0.01M.....	14.0	8.8	37
Pyruvate 0.01M.....	12.6	11.9	5

anaerobically to succinic acid is not clear (Weil-Malherbe, 1937a) and indeed, it seems very doubtful how any of the changes which pyruvic acid is so far known to undergo anaerobically can account for the aerobic oxidation of the ketonic acid. Krebs and Johnson (1937a) believe that the dismutation reaction which pyruvic acid undergoes anaerobically with brain tissue precedes the oxidation process, so that in effect the oxidation of pyruvic acid becomes the oxidation of lactic acid produced by the dismutation. This, however, is unlikely to be the case since Jowett and Quastel (1937a) have shown that hydroxymalonate inhibits *d*-lactate and *dl*-lactate oxidation by rat brain cortex slices to a much greater extent than pyruvate oxidation. This fact is shown in table 13.

The possibility that pyruvic acid is oxidized aerobically through succinic acid has been often considered (Toenniessen and Brinkmann, 1930; Elliott and Schröder 1934; Elliott and Greig, 1937; Weil-Malherbe, 1937b; Krebs, 1937) and undoubtedly the hypothesis is an attractive one. Quastel and Wheatley (1932), however, could not reconcile the hypothesis with the fact that there is no demonstrable formation of

l-malate when brain tissue is respiring in presence of pyruvate. The oxidation of *l*-malate is so slow compared with that of pyruvate by minced brain that some accumulation of *l*-malate should have occurred. These authors showed that with minced brain tissue succinate is oxidized quantitatively to *l*-malate, which can be estimated with fair accuracy by polarimetric means. McGowan and Peters (1937) disagree with the view that succinate is a necessary intermediate in the oxidation of pyruvate.

Whether pyruvic acid oxidation in brain can be adequately interpreted by some such cycle as the "citric acid cycle" of Krebs and Johnson (1937b) has still to be determined and at present it must be concluded that the main mode of aerobic breakdown of pyruvic acid in brain is unknown.

The action of vitamin B_1. A large literature has now accumulated on the action of vitamin B_1 on pyruvic acid metabolism, first discovered by Peters and his colleagues, and it will not be possible to deal with the phenomenon here in any detail. Reference may be made to a recent review by Peters (1937a) on this subject. The subject is also reviewed briefly by Silberschmidt (1936), where full references to the literature on the action of vitamin B_1 in general metabolism are given.

There seems to be no doubt that the brain tissue of pigeons suffering from avitaminosis B_1 respires at a smaller rate in presence of glucose or lactate than normally (Gavrilescu *et al.*, 1932) and that the addition of vitamin B_1 to the brain tissue *in vitro* restores the ability of the brain to respire at a normal rate. When such a brain respires in a lactate medium, pyruvate appears at considerably higher concentrations than with a normal brain (Peters and Thompson, 1934), and the addition of vitamin B_1 to the system causes this pyruvate to disappear. Moreover the minced brain tissue of an avitaminous B_1 pigeon respires at a lesser rate in a pyruvate medium than in such a medium containing added vitamin B_1. Pyruvic acid was the first substance proved to disappear as a result of the action of vitamin B_1. The phenomenon does not only apply to pigeon brain; it applies also to rat brain (O'Brien and Peters, 1935) and to chicken brain (Sherman and Elvejhem, 1936a).

Galvao and Pereira (1936) show that with rats suffering from avitaminosis B_1 the presence of lactate has less effect on the oxygen uptake by the cerebrum than is normally the case. The respiration of the brain stem is unaffected.

Galvao and Florence (1934) have found that the reduction of methyl-

ene blue by lactate is decreased from the normal rate when brain tissue of chickens suffering from avitaminosis B_1 is used.

The results of the investigations of the Oxford school of workers have led to the conclusion that vitamin B_1 is in some way specifically related to the oxidation of pyruvic acid (Peters, 1936; see also Sherman and Elvejhem, 1936b). This result has been amply confirmed by other workers (e.g., Lipmann, 1937; Westenbrink and Polak, 1937). Sinclair (1933) found that the respiratory quotient of brain tissue from polyneuritic pigeons is low and is raised nearly to the normal value by the addition *in vitro* of vitamin B_1.

The amount of vitamin B_1 in brain tissue has been calculated to be 1 unit per gram (Passmore, Peters and Sinclair, 1933).

The mode of action of vitamin B_1 is unknown. Its specificity of action was early shown by Gavrilescu *et al.* (1932) in the fact that succinate oxidation was normally oxidized by the brain tissue of a polyneuritic pigeon (confirmed by Galvao and Florence, 1934, for chicken brain). Krebs (1936) has stated that the vitamin is concerned with anaerobic breakdown of pyruvic acid but Peters (1936) could not confirm this statement.

The discovery by Lohmann and Schuster (1937) that cocarboxylase is a pyrophosphoric ester of vitamin B_1 led to the suggestion that the accumulation of pyruvic acid during the respiration of a polyneuritic pigeon brain is due to lack of this coenzyme. Peters (1937b) finds that although vitamin B_1 is rapidly phosphorylated by brain tissue, cocarboxylase has less effect than vitamin B_1 in increasing the oxygen uptake of polyneuritic pigeon brain. It is therefore unlikely, but it is not impossible, that vitamin B_1 exerts its effect on pyruvate oxidation as a phosphoric acid ester.

Oxidation of succinic acid in brain. This oxidation which, as has already been stated, is unaffected by the absence of vitamin B_1 from brain has been studied by a number of workers (Ashford and Holmes, 1930; Quastel and Wheatley, 1932; Peters and Sinclair, 1933). Succinic acid is rapidly oxidized by brain both aerobically and anaerobically (by methylene blue or by ferricyanide). It was shown by Quastel and Wheatley (1931) that the oxidation of succinate by brain proceeds to fumarate and *l*-malate, the further removal of the latter substance being relatively very slow. This has been confirmed by Elliott *et al.* (1937). The oxidation of succinate is independent or partially independent of the oxidations of glucose or lactate. The presence of malonate greatly inhibits the oxidation of succinate by brain. According to Greville

(1936) malonate brings about a decrease of the oxygen consumption of rat brain in presence of glucose, pointing possibly to succinic acid being involved in the respiration of glucose; the further addition of fumarate does not, however, completely neutralize this inhibition as should occur if the "succinic acid cycle" of Szent-Gyorgyi and his colleagues applies to brain respiration.

OXIDATION OF OTHER SUBSTRATES BY BRAIN. *l-Malate.* The addition of this substance to brain slices has a small effect in increasing the Q_{O_2} (see Elliott *et al.*, 1937). It is definitely oxidized in presence of brain slices anaerobically, by ferricyanide so long as cozymase is added to the system (Quastel and Wheatley, 1938).

α-Glycerophosphate. This substance reduces methylene blue in presence of brain tissue (Ashford and Holmes, 1931; Davies and Quastel, 1932).

Peters and Sinclair (1933) found that α-glycerophosphate is oxidized by minced pigeon brain, the mechanism being independent of lactate oxidation. β-Glycerophosphate is not oxidized by the tissue. Johnson (1936) has found that the aerobic removal of α-glycerophosphate in presence of minced pigeon brain is accelerated by the addition of sodium pyrophosphate. It is not influenced by the presence of pyruvate. Reducing substances are formed when α-glycerophosphate is oxidized by brain and from solutions in which this oxidation has proceeded the 2:4 dinitrophenylbis-hydrazone of methylglyoxal and the 2:4 dinitrophenyl hydrazone of a compound of probably six carbon atoms have been prepared. A study of α-glycerophosphate dehydrogenase has been made by Green (1936) who finds that in the rabbit the highest concentration of the enzyme is in the brain. The presence of α-glycerophosphate (0.03 *M.*) increases Q_{O_2} of rat brain cortex from 3.0 to 8.4, the effect being considerably less than that of glucose (Quastel, Tennenbaum and Wheatley, 1936).

Acetate. The addition of this substance has little or no effect on the respiration of brain (Elliott, Greig and Benoy, 1937; Weil-Malherbe, 1937a; Mann, Tennenbaum and Quastel, 1938).

Acetoacetate. Acetoacetate produces a small increase of the respiration of brain (McGowan and Peters, 1937; Krebs and Johnson, 1937; Mann, Tennenbaum and Quastel, 1938). It undergoes some reduction to β-hydroxybutyrate under anaerobic conditions (Weil-Malherbe, 1938).

β-Hydroxybutyrate. This substance is oxidized by rat brain cortex acetoacetate being formed (Jowett and Quastel, 1935).

α-Ketoglutarate. α-Ketoglutaric acid is oxidized by brain slices (Krebs and Johnson, 1937) and by minced pigeon brain (McGowan and Peters, 1937) but there is little or no accelerating effect of vitamin B_1 on its oxidation (in contrast to that with pyruvic acid) when polyneuritic pigeon brain is used. α-Ketoglutaric acid, like pyruvic acid, gives rise to succinic acid when incubated anaerobically with minced pigeon brain (Weil-Malherbe 1937b). It is reduced in brain to 1-α hydroxyglutarate.

α-Ketoadipate. α-Ketoadipic acid causes no change in the respiration of minced pigeon brain (McGowan and Peters, 1937).

Acetopyruvate. This substance is utilized both aerobically and anaerobically by pigeon brain (Krebs and Johnson, 1937c).

α-Hydroxyacetoacetate. This substance is not affected by rat brain slices (Weil-Malherbe, 1938).

Phosphoglycerate. Phosphoglyceric acid leads to a slight increase in the Q_{O_2} of guinea-pig brain cortex slices (Jowett and Quastel, 1937a). This fact is of interest since if phosphoglycerate behaves with brain as it does with muscle, it should be transformed to pyruvate and hence raise the respiration of brain tissue appreciably. This does not occur; nor does it raise the respiration in presence of α-glycerophosphate.

Gluconate. Sodium gluconate does not increase the respiration of minced guinea-pig brain tissue (Quastel and Wheatley, 1932).

Citrate. Sodium citrate was shown by Thunberg (1923) to be a hydrogen donator in presence of nerve tissue. It actively reduces methylene blue in presence of minced ox brain tissue (Davies and Quastel, 1932). Its Michaelis constant is shown in table 10. Its dehydrogenase is apparently very labile compared with that for succinate, lactate or α-glycerophosphate.

Hexosediphosphate, hexosemonophosphate (Robison ester), glycerol. These substances are oxidized by brain cortex slices but at relatively slow rates compared with that of glucose.

Alcohol. Robertson and Stewart (1932) have shown that ethyl alcohol in small concentrations may undergo oxidation in the presence of brain tissue; in large concentrations it acts as a narcotic presumably by displacing the metabolites present in the brain from the active surfaces.

Himwich and his colleagues (1933) have made observations of the R. Q. of the cerebral cortex of the rat after treatment with alcohol. The R. Q. was found to drop from a value of unity to an average value of 0.87.

Inositol. This substance does not increase the respiration of rat or rabbit brain (Young, 1936).

Fatty acids. Guinea-pig brain cortex slices are unable to oxidize lower fatty acids (Quastel and Wheatley, 1933).

Amino acids. Glutamic acid (0.05 M) is oxidized at a comparatively low rate by either fresh minced brain tissue or by tissue which has been allowed to respire for three hours before the amino acid is added (Quastel and Wheatley, 1932). Krebs (1935) has confirmed the fact that glutamic acid is oxidized by brain and has also shown that brain slices synthesize glutamine from glutamic acid and ammonia so long as energy is supplied by glucose oxidation in brain. Weil-Malherbe (1936) has found that whilst l (+) glutamic acid is burned by the brain, d (−) glutamic acid is not oxidized. Apparently l (+) glutamic acid is the only amino acid oxidized by brain. It is oxidized first to α-ketoglutaric acid, whose further breakdown can be inhibited by the addition of arsenite to the brain tissue. The Q_{O_2} of rat brain cortex is increased from 2.9 to 8.0 by the presence of 0.02 M l (+) glutamate (Jowett and Quastel, 1937b).

Amines. An amine oxidase exists in brain which brings about the oxidation of the aliphatic amines, butylamine, amylamine, isoamylamine and heptylamine. Propylamine, ethylamine and methylamine are not oxidized. This amine oxidase will also oxidize tyramine and indolethylamine (Pugh and Quastel, 1937) and adrenaline and various other aromatic amines (Blaschko *et al.*, 1937). The amines in question are oxidized by brain tissue to the corresponding aldehyde, ammonia being liberated. The activity of amine oxidase is inhibited by amines not oxidized by brain, e.g., ephedrine (Blaschko *et al.*, 1937), and by benzedrine (Pugh and Quastel, unpublished). The question has arisen as to whether some of the clinical effects of ephedrine administration are due to its inhibition of amine oxidase (see Gaddum, 1938).

It should be emphasized that no substrates have yet been found which equal either glucose, lactate or pyruvate in increasing and maintaining at a constant rate, the respiration of brain tissue *in vitro*.

Influence of the ionic environment on brain respiration. Ashford and K. C. Dixon (1935) have observed that the presence of 0.1 M KCl greatly increases lactic acid production in the presence of oxygen but decreases it in the presence of nitrogen. The total oxygen uptake is also increased by the presence of 0.1 M KCl. According to K. C. Dixon (1936) the addition of potassium chloride raises the aerobic destruction of glucose by brain to the anaerobic level. A careful investigation of

the phenomenon by Dickens and Greville (1935), working with rat brain cortex slices, shows that the presence of 0.1 M KCl increases the respiration of brain tissue whether glucose, lactate, pyruvate or fructose is the substrate. Moreover all the alkali metal chlorides increase the respiration of brain tissue when added in 0.1 M concentration to the medium. The order of activity is Li < Na < Rb < Cs < K. Results of a similar description have been obtained by K. C. Dixon and Holmes (1935). Dickens and Greville state that when brain gray matter is respiring in a salt medium the respiration is steadiest when Na, K and Ca ions are present. K^+ tends to increase the respiration, the bivalent cations Ca and Mg to decrease it. These changes in ionic concentration, which have large effects on brain metabolism, have no such effects on other normal tissues investigated—these being kidney cortex, testis, liver, yolk sac and retina.

TABLE 14

ANIMAL	Q_{O_2}	Q_{O_2} IN PRESENCE OF NARCOTIC
Guinea pig	3.9	0.37
Cat (grey matter)	2.8	0.27
Rabbit	2.8	0.47
Mouse	3.2	0.94
Calf (grey matter)	3.9	0.97
Sheep (grey matter)	3.2	0.80

EFFECTS OF DRUGS AND POISONS ON RESPIRATION IN THE CENTRAL NERVOUS SYSTEM. *Narcotics.* The large literature bearing on narcotic action cannot be dealt with here. It has been summarized by Winterstein (1926, 1936) and Henderson (1930), (see also Pick, 1937).

Bülow and Holmes (1932) have stated that the oxygen uptake of minced brain tissue is unaffected by the presence of anesthetic gases such as nitrous oxide and acetylene. It may be shown clearly, however, that the oxygen uptake of brain tissue is very considerably diminished by the presence of many different types of narcotics.

Table 14 shows the effects of a 0.4 per cent solution of somnifaine (a drug composed of the diethylamine salts of veronal and allylisopropyl barbituric acid) on the respiration of fresh minced brain tissue in a phosphate-saline (glucose free) medium (Quastel and Wheatley, 1932b).

Whereas the narcotics had highly inhibitive effects on the respiration of brain they had none at the concentrations used on an actively respiring yeast (see table 15).

The results of narcotics cited above have been found with minced brain tissue. They apply equally to brain tissue in the form of cortex slices. This is seen in table 16 showing the inhibitive action of chloretone (0.002 M) on the respiration of guinea-pig brain cortex slices.

The inhibitive effect of the narcotic is not due to irreversible damage of the brain tissue as shown by the fact that on washing the brain slices which have been exposed to the narcotic in a glucose phosphate medium the initial respiration of the brain is largely recovered. This reversi-

TABLE 15

NARCOTIC (0.3 PER CENT)	μL O_2 UPTAKE BY YEAST (0.05 GRAM) IN 2 HOURS	μL O_2 UPTAKE BY GUINEA PIG BRAIN (MINCED) 0.5 GRAM IN 2 HOURS
None	252	840
Ethylurethane	251	670
Allylisopropyl-barbituric acid	290	492

TABLE 16

Rate of O_2 uptake by guinea-pig brain cortex slices in a phosphate-saline-glucose medium in the absence and in the presence of chloretone (0.002 M)

	Q_{O_2}				AVERAGE Q_{O_2}	PER CENT DECREASE IN Q_{O_2} DUE TO NARCOTIC
	min.	*min.*	*min.*	*min.*		
Time from commencement of experiment	15	30	45	60		
In the absence of the narcotic	14.7	15.6	15.9	15.9	15.5	
In the presence of the narcotic	4.8	5.1	4.8	5.3	5.8	68

bility in action is shown in table 17 (Quastel and Wheatley, 1934). The following facts are noteworthy:

1. The Q_{O_2} of brain slices remains nearly constant throughout the experimental period.

2. The effect of the narcotic is rapid, the Q_{O_2} in presence of the narcotic reaching its equilibrium value within 15 minutes.

3. The Q_{O_2} does not fall progressively with time as the brain slices continue to be exposed to the narcotic. Were the narcotic a "cell poison" having irreversible action it would be expected that a progressive fall in respiration would occur.

4. The effect of washing brain slices previously immersed in the narcotic solution and of adding the washed slices to a fresh glucose

medium is to raise the Q_{O_2} to a level not far removed from that obtained with brain slices to which no narcotic had been added. With such washed brain slices a constant Q_{O_2} is rapidly reached showing the attainment of a new equilibrium.

Two-hundredths per cent (0.001 M) chloretone exercises an inhibition of the respiration of guinea-pig brain cortex slices of 50 per cent the inhibition being largely removed (to the extent of 70 per cent) by washing the slices. Eight-hundredths per cent luminal (0.0035 M) inhibits the respiration of cortex slices by 40 per cent the inhibition being decreased 80 per cent by washing the slices even after they had been exposed to luminal for 2 hours.

Wortis (1935) has used two methods to measure the effects of sedatives on oxidation by brain tissue:

1. Injection of different drugs into normal white rats was carried out until narcosis resulted. The animal's brain was then removed and the

TABLE 17

	Q_{O_2} IN PHOSPHATE GLUCOSE MEDIUM (AFTER WASHING THE CORTEX SLICES PREVIOUSLY EXPOSED TO CHLORETONE 0.002 M)							AVERAGE PER CENT DECREASE IN Q_{O_2}
	min.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	
Time from the commencement of the experiment	15	30	45	60	75	90	120	
No narcotic previously added (control)	14.4	15.5	14.9	13.8	13.8	14.2	14.2	
0.002M chloretone previously added (producing 68% drop in Q_{O_2})	10.0	12.3	13.3	11.9	12.2	11.9	12.0	16

respiration of the brain tissue measured manometrically. Morphine, butyl-bromallyl-barbituric acid and sodium amytal did not appear to affect R. Q. or O_2 consumption when the results were compared with normal brain tissue. This negative result is to be expected since, as has been pointed out, the effects of the narcotics are reversible. Immersion of brain tissue containing a narcotic (at a sufficiently low concentration) into a narcotic free medium results in a washing out of the narcotic and the consequent resumption of normal respiration.

2. Normal rat brain tissues (minced) was immersed in fluids containing various drugs at different concentrations and the R. Q. and the O_2 consumption were subsequently measured over a period of 2 hours. Cocaine, luminal, caffeine, sodiobenzoate and sodium bromide reduced the oxygen consumption of brain tissue.

Sherif (1930) has shown that cocaine, novocaine and urethane decrease the rate of oxygen consumption of nerve tissue, the first named being the most and the last the least active.

Quastel and Wheatley (1932b) as a result of investigation of the respiration of guinea-pig brain tissue in presence of various metabolites and narcotics came to the following conclusions:

1. Narcotics in general inhibit the oxidation by brain of glucose, sodium lactate and sodium pyruvate. They have some inhibitory power on the oxidation of glutamic acid. They do not inhibit the oxidation by brain of sodium succinate or of p-phenylenediamine.

2. Among narcotics of the same chemical type, those with the greater hypnotic activity have the greater inhibitive power.

It was suggested that a view of the mechanism of narcosis fitting most closely to the facts is that a narcotic is adsorbed from the blood at a

TABLE 18

SUBSTRATE	Q_{O_2} WITHOUT NARCOTIC	PER CENT INHIBITION OF Q_{O_2} FOUND WHEN 0.08 PER CENT LUMINAL IS PRESENT
Nil	2.89	5
Glucose 0.01M	12.20	55
d-Lactate 0.02M	13.55	35
Pyruvate 0.02M	11.10	27
Glutamate 0.02M	8.03	15
Succinate 0.02M	9.53	Nil

specific area or centre of the nervous system. Here it brings about a diminution of the ability of the cells constituting the nervous centre to oxidize pyruvic acid, lactic acid or glucose. The access—or activation of oxygen—is quite unimpaired as shown by the lack of effect of the narcotic on the oxidation of sodium succinate or p-phenylenediamine. Narcosis—or a depression of the normal functional activity of the nervous center in question may then ensue. The specific effects of narcotics will depend on the specificities of adsorption in various parts of the nervous system.

Jowett and Quastel (1937b) studying rat and guinea-pig brain cortex slices find that the respiration is inhibited by evipan, luminal, chloretone and ether in presence of glucose, sodium lactate and sodium pyruvate. Table 18 illustrates results obtained with rat brain cortex slices and 0.08 per cent luminal.

The sensitivity of brain respiration, as compared with that of other organs, to a narcotic is shown in table 19.

Jowett and Quastel found that the inhibition of respiration by narcotics tends to be independent of time when the potassium ion concentration of the medium is at the normal serum level, but that it increases rapidly with time when the potassium ion concentration is low (0.002 M). They also found that narcotics bring about inhibition of respiration by slices of liver, kidney and diaphragm, both when no substrates are added and in the presence of some substrate such as pyruvate. The effects of ether on brain cortex slice respiration are shown in table 20 (Jowett and Quastel, 1937c).

The inhibitory action of ether on the oxidation of glucose or of lactate has a large temperature coefficient ($Q_{10^\circ} = 6$, between 37° and 42°).

TABLE 19

Effect of 0.033 per cent evipan on respiration of guinea pig tissues in presence of glucose

TISSUE (SLICES)	Q_{O_2} ALONE	Q_{O_2} IN PRESENCE OF EVIPAN	EFFECT OF NARCOTIC
			per cent
Kidney cortex	15.2	15.95	+5
Liver	4.25	4.15	−2
Testis	8.65	7.25	−16
Spleen	7.7	6.4	−17
Brain cortex	14.2	9.5	−33

It is clear that ether, when compared with other narcotics such as the barbiturates, has much more pronounced irreversible effects. The inhibitory effect of ether on glucose oxidation by cerebral cortex at the anesthetic concentration is of the order of 10 per cent (Jowett and Quastel, 1937c).

Table 21 (Jowett, 1938) summarizes the results of Jowett and Quastel (1937b, c) and of Jowett (1938) on the effects of narcotics on brain cortex respiration, in the presence of glucose, at the concentrations required to induce narcosis.

The data in table 21 are sufficient to show that a definite inhibition of respiration is produced by concentrations of the order producing deep narcosis.

The facts given above, which show the specificity of inhibition, effected by narcotics on the respiratory processes of the central nervous

system, acquire a special significance when it is considered, as has already been pointed out, that carbohydrate breakdown occupies a dominant position in the metabolism of the central nervous system.

TABLE 20

ANIMAL	SUBSTRATE 0.02 M	PERCENT INHIBITION AFTER 80 MINUTES IN PRESENCE OF	
		(a) 0.05–0.055 M ether	(b) 0.10–0.11 M ether
Rat	Glucose	52	79
	Lactate	32	
	Fructose	32	
	Glutamate	18	50
	Succinate	0	
Guinea pig	Glucose	60	80
	Lactate	29	
	Pyruvate	29	
	Galactose		42
	α Glycerophosphate	0	
	Hexosediphosphate	0	

TABLE 21

NARCOTIC	ANIMAL	NARCOTISING CONCENTRATION (M)	PER CENT INHIBITION OF RESPIRATION DUE TO THE NARCOTISING CONCENTRATION OF NARCOTIC	RATIO: CONCENTRATION REQUIRED TO PRODUCE 15 PER CENT INHIBITION, DIVIDED BY NARCOTISING CONCENTRATION
Ethyl urethane	Rat	0.022	6	2.9
Magnesium ion	Rabbit	0.005*	12	1.2
Magnesium ion	Rat	0.005	13	1.2
Chloral hydrate	Rat	0.0013	10	1.6
Luminal	Rat	0.00079	15	1.0
Chloretone	Rat	0.0010	20	0.7
Evipan	Guinea pig	0.00062	17	0.9
Avertin	Rat	0.00106	31	0.4
Chloretone	Guinea pig	0.0010	32	0.4

* Excess above normal.

It appears probable from these results that narcotics bring about a local anoxemia in those parts of the nervous system where they are adsorbed, owing to their interfering with the oxidation of glucose at the nervous center in question. The access of oxygen however is quite un-

impaired. It has to be borne in mind that the inhibition of respiration to be expected during narcosis as a mean effect spread over the whole of the gray matter of the cerebral cortex is not necessarily large. The effects of narcotics on function are often localized, and the effects on oxidation in the brain may also be localized.

Gross and Pierce (1935) have examined the effects of morphine on the oxidation of glucose by the brain tissue of normal rats and of rats made tolerant to morphine by daily administration of the drug over a period of three weeks. They found that the degree of inhibition by added morphine of the respiration of brain tissue of tolerant animals in the presence of glucose was less than that which obtained in the case of non tolerant animals.

Hundhausen (1938) could find no difference between the inhibitive effects of chloralhydrate, or luminal on the respirations of brain cortex or brain stem.

Alexander and Cserna (1913) and Yamakita (1922) found very large decreases in oxygen utilization by the brain *in vivo* when narcosis was induced by ether (see also Lázló, Urban and Weissenberg, 1935). It is clear, however, from what has been said that large decreases in oxygen utilization by the brain as a whole are not necessarily to be expected when narcosis is induced. Local decreases, at particular parts of the nervous system, need not affect markedly the oxygen consumption of the brain as a whole.

Narcotics greatly inhibit anaerobic oxidation of glucose, fructose, mannose and lactate (Davies and Quastel, 1932) in presence of brain tissue. A competition takes place between the narcotic and substrate (lactate) for the enzyme system involved. Anaerobically, however, narcotics appear to have less inhibitive effects on oxidations by the brain than under aerobic conditions.

The pathological effects in the central nervous system following the administration of hypnotic drugs have been described by Mott, Woodhouse and Pickworth (1926).

Amines. Quastel and Wheatley (1933b) have found that amines, chiefly derived from tyrosine, tryptophane and leucine, inhibit oxidative processes in the brain *in vitro*. There are several similarities between the action of (certain) amines and that of narcotics on brain oxidation. While cadaverine, putrescine, neurine and ethylamine have little action (at the concentrations used) on the oxidations of glucose and sodium lactate, tyramine, β-phenylethylamine, and β-phenyl-β-hydroxyethylamine have relatively large effects. Mescaline (β:3:4·5-trimethoxy-

phenylethylamine), well known for its production of visual hallucinations, has similar large effects. Indole, skatole, isoamylamine have highly inhibitory actions but histamine is without effect. With the exception of tyramine none of the amines tested affects appreciably the oxidation of sodium succinate.

Studies with guinea-pig brain cortex slices show the inhibitory action of mescaline on respiration to be reversible (Quastel and Wheatley, 1934). No reversibility however was found with indole. This may mean that indole has an irreversible damaging effect on the brain cells but it seems as likely that the failure to find reversibility was due to inadequate washing of the brain cortex slices.

Pugh and Quastel (1937) have examined the effects of a number of amines on the respiration of rat brain cortex slices in a glucose medium

TABLE 22

Respiration of brain cortex slices in a glucose medium in presence of tyramine and indolethylamine

AMINE	ANIMAL	Q_{O_2} (OVER 1ST HR)		Q_{O_2} (OVER 2ND HR)	
		Amine absent	Amine present	Amine absent	Amine present
Tyramine 0.0052 M.	Rat	15.5	9.1	12.2	4.6
	Guinea pig	19.6	12.1	16.4	4.7
	Sheep	7.6	4.5	5.0	3.1
Indolethylamine 0.0066 M.	Rat	14.6	11.7	12.3	3.9
	Guinea pig	19.6	11.1	16.4	4.0
	Sheep	7.6	4.4	5.0	2.1

and, in addition to confirming the results already quoted, find that indolethylamine and benzedrine exert pronounced inhibitory actions. Table 22 summarizes some of their results.

It is of interest that many of these amines, which have such inhibitory effects on brain respiration in presence of glucose, should be oxidized by an enzyme (amine oxidase) present in brain as well as in other organs.

It is also of importance to record the fact that a number of amines—phenylethylamine derivatives—give rise to catatonia when injected into animals (De Jong and Baruk, 1930; De Jong, 1932; Noteboom, 1934). Whether such catatonic symptoms are related to a depression of respiration in some part of the nervous system is a problem still to be investigated.

Iodoacetate. This substance while inhibiting greatly the oxidation of glucose by brain has little effect on that of lactate. Krebs (1931) gives the following figures for rat brain cortex:

	GLUCOSE, 0.2%	*dl*-LACTATE, 0.2%	GLUCOSE, 0.2%	*dl*-LACTATE 0.2%
	M CONCENTRATION OF IODOACETATE			
	0	0	5×10^{-4}	5×10^{-4}
Q_{O_2} (over first 30 minutes)..	14.3	13.9	2.0	14.0

According to Quastel and Wheatley (1932) iodoacetate (2.5×10^{-4} M) inhibits the oxidation of glucose, fructose and mannose by guinea-pig brain but has little effect on that of *dl*-lactate, pyruvate, glutamate or succinate. Peters, Rydin and Thompson (1935) find that, with pigeon brain, iodoacetate diminishes the accelerating action of vitamin B_1 on pyruvic acid oxidation by avitaminous tissue. Peters (1936b) finds that dichlor-diethylsulphone also has an effect similar to that of iodoacetate.

dl-Glyceraldehyde. This substance (0.01 M) greatly inhibits the respiration of brain in presence of glucose, though it has little effect in absence of glucose (Mann *et al.*, 1938; Baker, 1938).

Fluoride. Fluoride inhibits glucose oxidation to a greater extent than that of lactate (Holmes, 1930) and according to Peters, Rydin and Thompson (1935) it inhibits the accelerating action of vitamin B_1 on pyruvic acid oxidation.

Cardiazole (metrazol, pentamethylenetetrazol). Cardiazole, which has has now come into prominence, as a convulsive reagent in the treatment of schizophrenia has according to Wortis (1938) no effect on the respiration of brain tissue *in vitro* when respiring in a glucose medium. Himwich, Bowman, Wortis and Fazekas (1938) have shown that during cardiazole convulsions, an anoxemia develops. During various stages of the convulsions the oxygen saturation of the blood falls to 42 per cent. Even in the first moments after the convulsions cease the oxygen tension of the arterial blood does not attain normal value. It appears, therefore, that the effect of the convulsions is to reduce the oxygen supply to the nervous system (see also Himwich *et al.*, 1937).

It is worth pointing out, at this juncture, that in the present most widely used active treatments of mental disorder, namely, prolonged narcosis treatment, insulin "shock" therapy, and cardiazole convulsive treatment, there appears to be a common factor—an interference with

the respiratory metabolism of the central nervous system. In narcosis treatment there is probably a diminished ability to burn glucose at those nervous centers affected by the narcotic; in insulin "shock" therapy there is a diminished quantity of glucose available for all the nervous system, and in cardiazole convulsion therapy, the access of oxygen itself to the nervous system is cut down.

p-Aminonaphthol. This substance at a concentration of 10^{-4} M inhibits brain respiration by 40 per cent (Bernheim *et al.*, 1937).

Dinitro-o-cresol. Dinitrophenols increase the respiration of normal tissues (Dodds and Greville, 1933; Ehrenfest and Ronzoni, 1933). Greville (1936) describes an experiment showing that the Q_{O_2} of rat brain cortex in presence of glucose is raised from $Q_{O_2} = 13.1$ to $Q_{O_2} = 39.0$ by the addition 10^{-5} M dinitro-o-cresol. The addition of malonate abolishes the accelerating action of dinitro-o-cresol.

Pyocyanine. Young (1937) has shown that the presence of pyocyanine (10^{-3} M – 10^{-5} M) increases markedly the respiration of rat brain cortex in presence of glucose. Oxidation in presence of lactate, fructose and pyruvate are also increased, but when no substrate is added no increased respiration is noted.

Dyestuffs. The accelerative actions of dyes such as thionine, brilliant cresyl blue and the inhibitive action of other dyestuffs on brain cortex respiration in presence of glucose have been described by Dickens (1936). Elliott and Baker (1935) have also contributed to this subject, making use of 2-6-dichlorophenolindophenol and dinitrocresol.

Fractions from B. aertrycke. Toxic fractions from *Bact. aertrycke* have the power of greatly increasing the oxygen uptake of rabbit brain suspensions in the presence of glucose, but they have no effect in the absence of added substrate (Delafield and Smith, 1936). These toxic fractions slightly diminish the O_2 uptake of brain suspensions in presence of succinate.

Lead. According to Dolowitz, Fazekas and Himwich (1937) 5 mgm. lead acetate per 100 grams tissue reduce the oxygen consumption of brain tissue *in vitro.*

Acetylcholine synthesis in the central nervous system. It has been shown recently (Quastel *et al.*, 1937; Mann *et al*, 1938) that acetylcholine synthesis in the brain depends upon the existence of an intact respiratory metabolism. Under anaerobic conditions the synthesis of this substance does not take place, and under aerobic conditions either glucose, lactate or pyruvate is essential for the formation of acetylcholine to occur.

Content of vitamins concerned with respiration and of glutathione in the

central nervous system. *Vitamin* B_1. The content of this substance in the brain has already been mentioned.

Lactoflavin (*vitamin* B_2). This vitamin is present in brain and its amount is given as 1γ–5γ per gram in beef brain (Euler and Adler, 1934), as 2.2γ per gram in gray matter of sheep brain and as 1γ per gram in the white matter (see Gourévitch, 1937). Charite and Khaustov (1935) state that human brain contains 1.5γ per gram. Neurological manifestations in vitamin B_2 deficiency are described by Zimmermann *et al.*, 1937.

Ascorbic acid. Bessey and King (1933) give the following values of the ascorbic acid content of brain. Rat 0.36 mgm/gram; rabbit 0.31 mgm/gram; chicken 0.33 mgm/gram; hog 0.18 mgm/gram. Plaut and Bülow (1934, 1935) find brain rich in ascorbic acid and quote the figures given in table 23, which show the variation of ascorbic acid content of various parts of the brain with age.

TABLE 23

	NUMBER OF CASES	AGE	ASCORBIC ACID IN		
			Cortex	Cerebellar cortex	Spinal cord
			mgm. per cent	*mgm. per cent*	*mgm. per cent*
Fetus	7	3–5 months	65	37	16
Infant	6	1½–9 months	23	31	17
Children	4	2½–8½ years	18	23	16
Adult	16	25–56 years	10	13	13
Aged	12	65–82 years	7.5	9	11

Wortis, Wortis and Marsh (1938) have recently confirmed the finding of Plaut and Bülow (1936) that ascorbic acid in the cerebrospinal fluid is an accurate index of the amount present in the blood. They find that the R. Q. of brain of scorbutic guinea-pig brain is unity and that such brain respires at the same rate as the normal. Gluck and Biskind (1935), studying the pituitary, find that the concentrations of ascorbic acid in the pars nervosa, intermedia and distalis are in the ratio 1:4.7:3.

The details and mechanisms of action of lactoflavin or of ascorbic acid in the respiratory processes of the nervous system are at present unknown.

Glutathione. Glutathione occurs in rabbit brain to the extent of 75 mgm. per cent wet weight in gray matter and 52 mgm. per cent in white matter (Holmes, 1926). Possibly part of this is actually ascorbic

acid since iodine titrations were used. Boyland (1933) gives the glutathione content of rat brain as of the order 50 mgm. per cent whilst according to Wachholder, Anders and Uhlenbrook (1935) the glutathione content of rabbit brain is 63–95 mgm. per cent.

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CELLULAR OXIDATION SYSTEMS

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Combustion of foodstuffs through oxygen was Lavoisier's (207) conception of respiration. It is still ours, and for this reason the term *oxidation* has been retained in this review. Experiments since Lavoisier's time have discovered the intermediate steps between foodstuff and oxygen. Foodstuffs, which *in vitro* are oxidized only by powerful oxidants or under conditions which do not exist in biological systems, are oxidized in living cells at relatively low temperatures, at neutrality, through the action of mild oxidizing agents, the oxidizing enzymes. Thus cellular respiration, the consumption of oxygen accompanied by the production of CO_2 and H_2O, represents the integral of a number of partial oxidation-reductions, each of which has its peculiar mechanism. The work of the past ten years on the isolation of a number of oxidation enzymes and on the kinetic and energetic aspects of biological oxidations has given us a clearer understanding of the process of cellular respiration. It must be admitted that the fate of the simplest foodstuff, glucose, when it is used by living cells in the process of respiration, seems to preclude any possibility of a unified concept of cellular respiration. For glucose is directly oxidized by some cells; in others it is first split by glycolysis, the split products being partially oxidized in a few steps, all irreversible; in still others the split products of glycolysis are oxidized also in thermodynamically reversible steps introduced by enzymes; and, finally, in some cells glucose is oxidized to completion by the aid of a series of reversible oxidation-reduction systems of graded potential up to molecular oxygen, the energy being thus released gradually. In all these cases, however, respiration takes place by a transfer of electrons from the foodstuff through the oxidation catalysts to molecular oxygen. In the simplest cases the transfer is made by only one reversible system; in the more complicated cases the electron transfer is carried on step by step through a series of reversible oxidation-reduction systems, which regulate the release of energy, like locks in a canal, and prevent its sudden wasteful release. We may then conceive

of cellular respiration simply as the process by which the cell extracts energy from foodstuffs, this release of energy depending upon the transfer of electrons from the foodstuffs, first to systems capable of receiving them directly, and from them, by a series of oxidation-reduction systems, to atmospheric oxygen.

The conditions which make possible and regulate a determined reaction are two: energetic and kinetic. It is essential in the first place for the reaction to be thermodynamically possible. Afterwards we must search for the path it follows. The possibility of a reaction is determined by the knowledge of the equilibrium, concentrations of reactants, and products of the reaction. The data thus obtained are accurate, trustworthy. The path followed by the reaction, i.e., its mechanism, can be approached by studying the rate of reaction, its relation to the concentration of reagents, the influence of different substances which either inhibit or accelerate it, the effect of temperature, etc. The difficulties surrounding the kinetic aspect of the problem are obviously manifold and the answers obtained not so precise. But only through the understanding of the problem of equilibrium reactions (energetics) and that of the rate of these reactions (kinetics) can the problem of cellular respiration be approached with reasonable probability of success.

I shall, therefore, pass in review: first, studies related to the possibility of reactions, i.e., thermodynamic studies; second, studies related to the mechanism of reactions.

OXIDATION-REDUCTION SYSTEMS OF BIOLOGICAL IMPORTANCE. Through the pioneering efforts of Clark and his collaborators, it is now possible to treat the subject of biological oxidations in a broad manner, for the fruitlessness of the discussions about whether biological oxidations are characterized by the addition of oxygen or the withdrawal of hydrogen was pointed out by Clark in 1923 (59). Clark at that time gave the following expression to a definition of oxidation that had had a long evolution: "The withdrawal of electrons from a substance with or without the addition of oxygen or elements analogous to oxygen; or as the withdrawal of electrons with or without the withdrawal of hydrogen or elements analogous to hydrogen."

If a reaction under investigation can be made to take place in a reversible galvanic cell, the maximum electromotive force of the cell may be measured by the potentiometric method and the free energy change of the reaction is then given by the equation,

$$\Delta F = -nFE,$$

where n is the number of equivalents of chemical change involved in the reaction equation, F is Faraday's constant (23,068 calories, when ΔF is to be expressed in calories), and E is the electromotive force of the cell.

When this simple method of determining free energies was introduced into biology by Gillespie (129) and Clark, it was found empirically that some thermodynamically reversible systems of biological importance behave unusually towards noble metal electrodes. And after years of toil it was recognized that besides those systems where their electron charges come immediately into equilibrium with those of a noble metal electrode, there are systems where the potentials can be obtained only on the addition of an electroactive substance. Furthermore, there are systems where the potentials are obtained only in the presence of an enzyme and an electroactive substance. The reason for this difference of behaviour is not known. Those belonging to the first group are the *electromotively active systems*; those belonging to the second group are the *sluggish systems*; those belonging to the third group are the *enzymatic-sluggish systems*.

Electromotively Active Systems. Electromotively active oxidation-reduction systems are those which, like $Fe^{++} \rightleftharpoons Fe^{+++} + e$, can exchange electrons directly with a noble metal electrode. As a rule, they are autoxidizable, i.e., readily oxidized by atmospheric oxygen. The free energy of these systems can be easily determined by the potentiometric method, the use of which was greatly stimulated among biologists by the studies of Clark and his co-workers, and by the publication of Michaelis' excellent monograph on the subject (227).

The free energies of these systems, i.e., the oxidation-reduction potentials, are determined directly by measuring with the potentiometer the E.M.F. given by two half cells, the standard and that of the system under study, and applying the equation

$$E_h = E_0 - \frac{RT}{nF} \ln \frac{[S_r]}{[S_0]} - \frac{RT}{nF} \ln K,$$

where E_h is the observed E.M.F. referred to the hydrogen electrode, E_0 is a constant involving all those constants which can be so combined, $[S_r]$ and $[S_0]$ the concentrations of total reductant and oxidant respectively, and K, a constant, function of H^+ and equilibrium constants.[1]

[1] For a derivation of this equation from the mass law see Clark and Cohen (65); for a thermodynamic derivation see Lewis and Randall (212).

Iron-porphyrin compounds.[2] The iron-porphyrin compounds occupy a unique position among the oxidation-reduction systems of biological importance, because on combining with nitrogenous compounds they give complexes possessing a variety of properties. Thus, when certain iron-porphyrins combine with a protein they form compounds (hemoglobin, myoglobin, erythrocruorin) which can combine with molecular oxygen reversibly, like the Werner complexes, with no electronic exchange; iron-porphyrins may combine with proteins and give non-autoxidizable sluggish oxidation-reduction systems (cytochrome *c*); finally, they may combine with proteins or other nitrogenous compounds giving autoxidizable, electroactive systems (hemochromogens, some of the cytochromes).

The properties of the first class of iron-porphyrin compounds (hemoglobin, myoglobin) will not be discussed in this review, although their respiratory function as oxygen storehouses is indeed of first importance in cellular respiration. Discussion will be limited to the last two groups, those able to act as electron mediators, which are directly concerned in oxygen utilization.

The classical paper of Keilin in 1925 "On cytochrome, a respiratory pigment, common to animals, yeast and higher plants" (166), and the elegant demonstration of Warburg and Negelein in 1928 (315) of the relative spectrum of their Atmungsferment, contributed to re-awaken the interest of investigators in iron-porphyrin compounds, the importance of which had been neglected since MacMunn's pioneer studies (see Anson and Mirsky's review (9)).

The universality of the distribution of iron-porphyrin compounds was recognized as early as 1886 (MacMunn (220)). They have been found in all aerobic bacteria (Yaoi and Tamiya (335); Tamiya and Yamagutchi (285), Yamagutchi (334); Frei, Riedmüller, and Almasy (110)); in plants (Kempner (178)); in lower animals (Roche (259)); in higher animals (Cohen and Elvehjem (68), Huszak (160)). They have been found even in organisms such as paramecium (Sato and

[2] To avoid confusion, the nomenclature used in this review will be as follows: *Iron-porphyrin* is used as a class name; *hemin* is used to designate the FeCl compounds of porphyrins, with a prefix designating the kind of porphyrin (blood hemin; spirographyshemin, pheohemin); *hemochromogen* is used for the nitrogenous coördination compounds of iron-porphyrin, the prefixes ferri- and ferro- indicating whether these compounds are oxidized or reduced—e.g., ferri- and ferro-nicotine hemochromogen. The word cytochrome has been retained because the nitrogenous compound bound to the iron-porphyrin nucleus is still unknown.

Tamiya (262)), whose respiration is insensitive to HCN (Gerard and Hyman (125)).

The property of iron-porphyrins and of their nitrogenous coördination compounds of possessing strong absorption spectrum bands, and that of some of these last compounds of combining reversibly with HCN and CO have been extensively used for their detection in living cells.

In table 1 are given representative examples of the position of the maximum absorption spectrum bands of hemochromogens, Keilin's cytochromes, as found in tissues and isolated cells. A comparison of these absorption bands to those obtained in solutions of iron-porphyrins and hemochromogens of known chemical constitution makes plausible the suggestion of Keilin (168) that the "cytochromes" found in living cells may be grouped in three classes: *a*, those with absorption bands

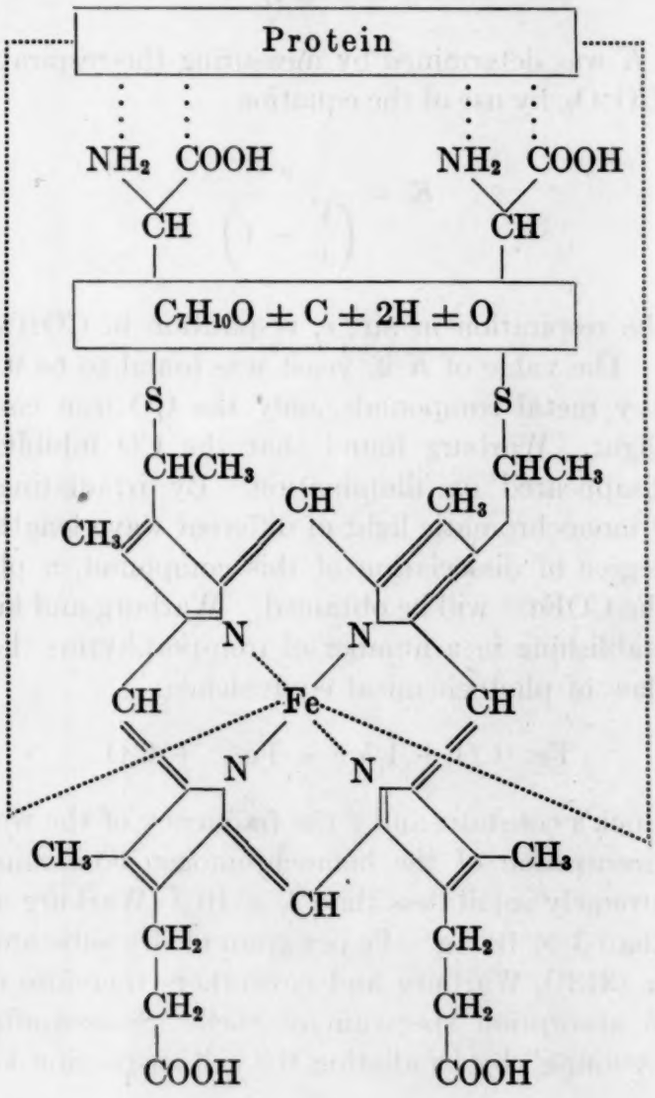
TABLE 1

Position of the absorption spectrum bands of ferrohemochromogens present in cells (cytochromes)

	I	II	III	IV	V
Bee-wing muscles	6048	5665	5502	5210	4490; 4330; 4170
Guinea pig; heart muscle	6045	5662	5500	5205	?
Brewer's yeast	5860	5650	5510	5250	
Baker's yeast	6030	5650	5500	5230	
B. coli	6285; 5900	5600		5300	
B. pasteurianus	6350; 5890	5680	5520	5270	
Proteus mirabilis	6310; 5950	5600		5300	

around I, IV, and V; *b*, those with bands around II, IV, and V; and *c*, those with bands around III, IV, and V. The properties of these cellular hemochromogens have been described in detail by Keilin (168), Reid (256), and Shibata (268) in recent reviews. The chemical properties of cytochromes *a* and *b* are not yet known, although some work has been done by Roche and Benevent (262), who believe that their "heme *a*" as prepared from heart muscle has spectral properties similar to those of Keilin's cytochrome *a*, and by Yakushiji and Mori (332), who claim to have prepared natural and synthetic cytochrome *b*. The authors claim that both preparations are autoxidizable. More is known about cytochrome *c* as it has been prepared in pure form by Keilin and Hartree (172) and Theorell (289). Of these properties, the most important from the point of view of cellular respiration is that cytochrome *c* is not oxidized by measurable rate by atmospheric oxygen

in neutral solutions nor does it combine with HCN or CO at the concentrations used for respiration inhibitions (Altschul and Hogness (5) maintain that cytochrome *c* combines with CO). Cytochrome *c* is rapidly oxidized by Keilin's "cytochrome oxidase" (Keilin and Hartree (173)) and some hemochromogens (Mori, Okunuki, and Yakushiji (235)). The chemical constitution of cytochrome *c* has been studied by Theorell (293). According to him cytochrome *c* is made up of a porphyrine polypeptide complex containing two atoms of sulphur. The sulphur-containing side chain is bound to the porphyrin through both S atoms as in a thio-ether linkage. The protein component is bound to the side chain through peptide linkages of one or more of its amino and carboxyl groups; it is also bound to the Fe atom.



Heavy metals (Cu, Fe) combine with CO reversibly.

$$Fe^{++} + CO \rightleftharpoons Fe^{++}\,CO$$

Warburg (302) found that the respiration of yeast was inhibited by CO reversibly. During this process, with the assumption that the inhibition was due to the formation of a Fe^{++} CO compound, and that the remaining Fe^{++} compound is immediately transformed into Fe^{+++} compound by O_2, the ratio $\frac{Fe^{+++}}{Fe^{++}CO}$ depends on the $CO:O_2$ ratio according to equation

$$\frac{Fe^{+++}}{Fe^{++}CO} \times \frac{(CO)}{(O_2)} = K.$$

The value of K was determined by measuring the respiration at different ratios of $CO:O_2$, by use of the equation

$$K = \frac{r}{\left(\frac{V}{v} - 1\right)}$$

where V is the respiration in air; v, respiration in $CO:O_2$; and r, the ratio $CO:O_2$. The value of K in yeast was found to be 9.

Of the heavy metal compounds, only the CO iron compounds are sensitive to light. Warburg found that the CO inhibition of yeast respiration disappeared on illumination. By irradiating a $COFe^{++}$ compound by monochromatic light of different wave lengths and determining the degree of dissociation of this compound, a photochemical spectrum of the $COFe^{++}$ will be obtained. Warburg and his co-workers started by establishing in a number of iron-porphyrins the validity of the Einstein law of photochemical equivalence:

$$Fe^{++}CO + 1\,h\,v = Fe^{++} + CO,$$

where h is Planck's constant and v the frequency of the wave sent out. Since the concentration of the hemochromogen combining reversibly with CO is extremely small: less than 4×10^{-7} (Warburg and Negelein (316)), more than 3×10^{-8} g. Fe per gram of dry substance (Warburg and Kubowitz (313)), Warburg and co-workers therefore obtained the photochemical absorption spectrum of their "Sauerstoffübertragende Ferment der Atmung" by irradiating the cell suspension kept in a gas

phase of $CO:O_2$ with monochromatic light of different wave lengths (303).

The discovery of Anson and Mirsky (7) that hemochromogens are reversible compounds of iron-porphyrin and nitrogenous substances,

$$Fe^{+++} + 2\,N \rightleftharpoons Fe^{+++}N_2$$
$$Fe^{++} + 2\,N \rightleftharpoons Fe^{++}N_2$$

where N represents a nitrogenous substance, has been of first importance for the understanding of the properties of these substances. Ferri- and ferro-porphyrins combine as a coördination compound (Pauling and Coryel (248)) with 2 molecules of a nitrogenous substance, Hill (153), Langenbeck (206), Hogness et al. (156)) their equilibrium constants depending on the nature of the nitrogenous substance, hydrogen ion concentration, and temperature; furthermore, the coördination linkage may be made with two different nitrogenous compounds (Anson and Mirsky (8), Drabkin (88)). This great affinity of iron-porphyrins for nitrogenous compounds favors the opinion that they must always be present in living cells as hemochromogens; it also admits the possibility of rapid change from one hemochromogen into another through a change in the concentration of the nitrogenous substances and other factors which determine the value of the equilibrium constants.

Very little is known about the constitution of Keilin's cytochrome oxidase (indophenol oxidase). The enzyme is so intimately bound to the insoluble material of the cell that it becomes difficult to apply the ordinary methods of purification and isolation. It acts as a catalyst for the oxidation of cytochrome *c*, its catalytic effect being inhibited by HCN, H_2S, NaN_3, and CO (Keilin and Hartree (172)). Because of these inhibitions the belief has been widespread in the identity of Keilin's cytochrome oxidase and Warburg's "Sauerstoffübertragende Ferment der Atmung." It has been suggested that the enzyme may be a protein combined to cytochrome *a* or one of its components. Keilin and Hartree (173) present now strong arguments against this opinion and offer two other possibilities: the enzyme may be 1, an iron-protein compound devoid of porphyrin; 2, a copper-protein compound. The problem as to the nature of this enzyme remains open for further investigation.

Conant and his collaborators (71, 72) were the first to demonstrate that both the system ferrohemin-ferrihemin and the system ferrihemochromogen-ferrohemochromogen are electromotively active reversible oxidation-reduction systems, a one-electron transfer being involved in

the process. For blood hemin (26) the potential of the system at 30° is obtained from the equation

$$E'_h = E'_0 - 0.06 \log \frac{[Fe^{++}\text{hemin}]}{[Fe^{+++}\text{hemin}]} - 0.06 \log \frac{1}{H^+}$$

Where E_0 (the normal potential of the system at pH 0) in phosphate buffer was calculated to be +0.306 volt, and in borate buffer, +0.263 volt. The potentials of hemin became more positive on addition of a nitrogenous compound, the rise in potential being in inverse relation to the affinity of the nitrogenous compound for hemin. It was also found that when the affinity of the nitrogenous compound for hemin was great, as in the case of cyanide hemochromogen, the potential was independent of the hydrogen ion concentration; when the affinity was low, as in nicotine, α-picoline, and pyridine hemochromogens, the E_h values increased, as in hemin, by 60 millivolts per pH unit. The values of the potentials of spirographis hemin and some of its hemochromogens (Barron (27)) are more positive than those of blood hemin and its hemochromogens. All these hemochromogens are autoxidizable. Clark and his co-workers (67) have given a clear quantitative treatment of the empirical findings. By introducing the dissociation constants of the nitrogenous metallo-porphyrin compounds into the electrode equations, they have shown that if the affinity of the nitrogenous compound for the oxidized and reduced metallo-porphyrin is the same, there will be no change of potential on addition of the nitrogenous base. If the potential becomes more positive it means that the reductant forms the more stable compound; if the oxidant associated more strongly than the reductant, the trend of potential will be in the negative direction. Clark's treatment becomes of essential biological importance because of its possible extension to those numerous oxidation-reduction systems where the active nucleus (alloxazins, pyridinium nucleotides, and possibly thiamin) combines reversibly with nitrogenous substances.

The potentials of cytochromes will be discussed here only because of convenience, for some of these systems seem to act not as electroactive but as sluggish systems. At least, this is so in the case of cytochrome *c*, where attempts to titrate it according to Clark's methods for electroactive systems have failed (Green (133)). The potentials of the three cytochromes, *a*, *b*, and *c*, have recently been given in a short note by Ball (17). His values for cytochrome *c* are in fair agreement with those previously found by Coolidge (73) and Wurmser and Filitti-Wurmser (328). (These values for the potentials of cytochrome *c* have been

confirmed by Stotz et al. (280).) In table 2 are given the potentials (E'_0 values) at pH 7.0 of the cytochromes and of the iron-porphyrins and hemochromogens. One active nucleus, iron-porphyrin, because it combines reversibly with a number of nitrogenous substances, can alter its free energy so widely as to give the clearest example of a series of oxidation-reduction systems of graded potentials.

Alloxazins. (Two reviews have recently been published by Theorell (292) and Wagner-Jauregg (300).) The progress of our knowledge of the chemical constitution and properties of alloxazin has developed

TABLE 2

Oxidation-reduction potentials of hemins and hemochromogens

E'_0 values at pH 7.0, temp. 30°

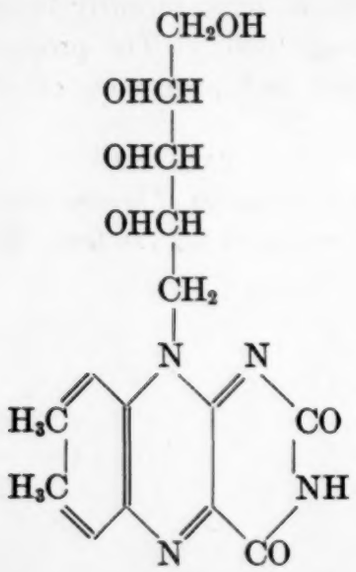
SYSTEM	E'_0
	volts
Blood hemin (phosphate)	−0.110
Nicotine hemochromogen	+0.184
Pyridine hemochromogen	+0.172
α-Picoline hemochromogen	+0.136
Histidine hemochromogen	−0.106
Globin hemochromogen	−0.098
Cyanide hemochromogen (pH 8.2)*	−0.183
Spirographis hemin	−0.077
α-Picoline spirographis hemochromogen	+0.155
Pilocarpine spirographis hemochromogen	−0.024
Cyanide spirographis hemochromogen (pH 8.2)*	−0.113
Cytochrome a pH 7.4	+0.290
Cytochrome b pH 7.4	−0.040
Cytochrome c pH 7.4*	+0.255

* Potentials independent of pH.

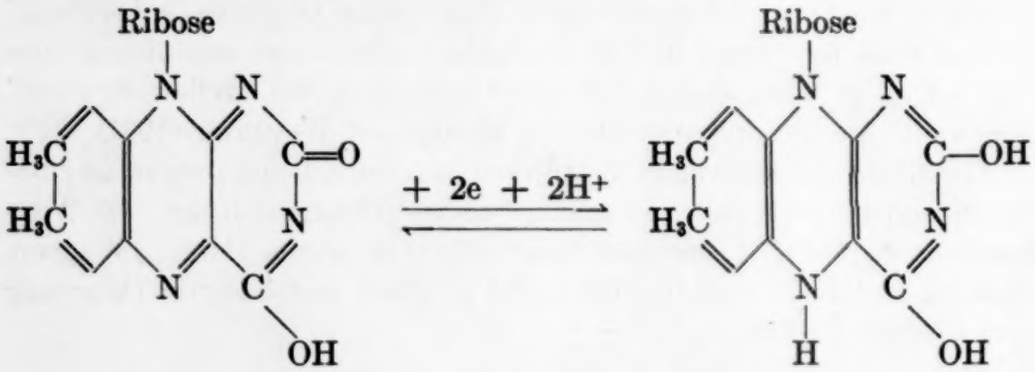
with extraordinary speed. Announced in 1932 as a new respiration enzyme, Warburg and Christian's (305) "gelbe Oxydations Ferment" in less than five years had its chemical constitution established, and but for its protein fraction we could have said the "yellow ferment" was synthetically prepared (Kuhn, Rudy, and Weygand (198)).

The alloxazins are widely distributed in nature, being present as riboflavin and its derivatives in animal tissues (Györgyi, Kuhn and Wagner-Jauregg (143), Euler and Adler (96)), in plants (Kuhn, Wagner-Jauregg and Kaltschmidt (200)), and in yeast and bacteria (Warburg and Christian (306)).

Riboflavin (6,7-dimethyl-9-d-ribityl-isoalloxazin) is a derivative of the hypothetical isoalloxazin, the yellow color of the pigment being due to the ring system of isoalloxazin. Kuhn and Wagner-Jauregg (199) were the first to show that the pigment was reversibly oxidized and reduced (although Warburg had previously found this property in his Yellow Ferment) and that in acid solutions the reduction of the

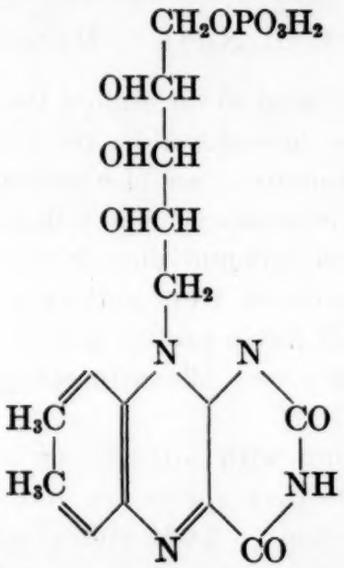


pigment proceeded through the intermediate formation of a semiquinoid radical. Riboflavin, as well as alloxazins, is an electroactive oxidation-reduction system. (The potentials of riboflavin and their derivatives have been measured by Klemperer, Bessy and Hastings (185), Kuhn and Moruzzi (197), Kuhn and Boulanger (195), Michaelis, Schubert, and Smythe (233), Stare (272), Stern (274).) Michaelis and his co-workers have found that the maximum amount of semiquinone formed at pH values of physiological significance is about 10 per cent. The reversible oxidation-reduction may be represented:

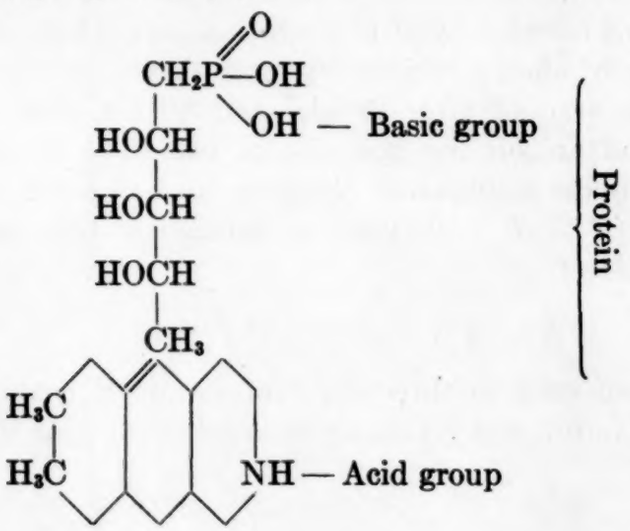


(For the structure of alloxazins in their various forms of oxidation-reduction and ionization, see Michaelis, Schubert, and Smythe (232).)

Riboflavin can be phosphorylated enzymatically by animal tissues (intestine) under the action of phosphatase (Rudy (261)), giving phosphoriboflavin:



Phosphorylation is also brought about by the adrenal cortex (Verzar, Hübner, and Lastz (297)). Phosphoriboflavin on combining with a protein gives phosphoriboflavin protein, with a molecular weight of 73,000 on the assumption that the compound contains one molecule of riboflavin. Kekwick and Pedersen (177) from measurements of the sedimentation velocity and diffusion constant give 80,000 as the molecular weight. The combination of flavin with protein is a reversible one as long as the protein component is not denatured. Theorell (291) and Kuhn et al. (195) have sggested that the basic protein group combines with the phosphoric acid group and the acid protein group with the basic imino group of riboflavin.



Warburg and Christian (310–311) have isolated from d-amino acid oxidase an alloxazin-adenine-dinucleotide which is formed by the union of riboflavin phosphoric acid and adenilic acid:

$$C_{17}H_{21}N_4PO_9 + C_{10}H_{14}N_5PO_7 - H_2O = C_{27}H_{33}N_9P_2O_{15}$$

This alloxazin has been found in all animal tissues and yeast, and like the other alloxazin is an autoxidizable, reversible oxidation-reduction system; it is also, presumably, an electroactive system. Ball (18), working in Warburg's laboratory, has isolated from milk xanthine oxidase, another alloxazin-adenine-dinucleotide. Corran and Green (74) have reported the isolation from milk of a new phosphoriboflavin-protein; whether this milk flavin-protein is part of the prosthetic group of xanthine oxidase or is a new alloxazin compound has not yet been established.

As hemin on combining with nitrogenous substances increases its potential toward more positive values, so, too, does phosphoriboflavin on combining with a protein (−0.061 volt at pH 7.0, 38°) (Kuhn and Boulanger (195)). Following the analogy, it is plausible to assume that different flavin-proteins may have different potentials.

Other oxidation-reduction systems. Besides these two systems whose rôle as catalysts of oxidations has been established, there have been found in biological fluids a number of substances which are electromotively active, and have been considered accessory respiration catalysts. (In table 3 are given the E'_0 values of these systems at pH 7.0.)

Michaelis two-step oxidation. (Michaelis has written three excellent reviews (228–230).) The discovery of Michaelis and Elema that pyocyanine (a phenazine dye) in acid solutions was reduced in two steps with the formation of an intermediate compound or semiquinone has attained considerable importance because of the ever-increasing number of systems being found in living cells which show semiquinone formation. First found only among cationic systems they were later also found among anionic systems after Preisler and Shaffer (252) reported the first example of anionic dye reducing in two steps in highly alkaline solutions. Suppose a substance R which can be reversibly oxidized to a substance $T(R \rightleftharpoons T + 2\ e)$ in two successive steps, each involving the loss of one electron:

$$R \rightleftharpoons S + e; S + e \rightleftharpoons T + e.$$

The system can exist in three different forms: R (reduced form), S (semi-oxidized form), and T (totally oxidized form). As the S form can

dismute reversibly: $2\,S \rightleftharpoons R + T$, the equilibrium k of the dismutation will be:

$$k = \frac{[R][T]}{[S]^2}$$

where k is Michaelis' dismutation constant; its reciprocal value $K = \frac{1}{k}$ is what he calls semiquinone formation constant. When a solution

TABLE 3

Oxidation-reduction potentials of electroactive systems present in biological fluids

E_0' values at pH 7.0

SUBSTANCE	ORIGIN	E_0'	TEMP.	ELECTRON TRANSFER	REFERENCE
		volts	°C.		
Adrenalone	Animal	+0.489	30	2 by 2 (?)	Ball, Chen, and Clark (19)
Epinephrine	Animal	+0.389	30	2 by 2 (?)	Ibid.
Homogentisic acid	Animal	+0.260	30	2 by 2 (?)	Fishberg and Dolin (108)
Phoenicein	Mold	+0.047	20	2 by 2 (?)	Friedheim (113)
Juglone	Plant	+0.036	20	2 by 2 (?)	Ibid. (116)
Pigment from *Arion rufus*		+0.025	20	2 by 2 (?)	Ibid. (112)
Hallachrome	Animal	+0.022	20	1 by 1	Ibid. (114)
Pyocyanine	Bacteria	−0.034	30	1 by 1	Friedheim and Michaelis (117); Elema (89)
Hermidin	Plant	−0.030		2 by 2	Cannan (56)
Toxoflavin	Bacteria	−0.049		1 by 1	Stern (275)
Phosphoriboflavin-protein	Yeast	−0.061	38	1 by 1	Kuhn and Boulanger (195)
Chromodoris zebra pigment	Plant	−0.102	30	1 by 1	Preisler (251)
Chlororaphin	Bacteria	−0.115	30	1 by 1	Elema (90)
Lawsone	Plant	−0.139	20	1 by 1 (?)	Friedheim (116)
Phthiocol	Bacteria	−0.179	30	1 by 1	Ball (13); Hill (152)
Lapachol	Plant	−0.180	30	1 by 1	Ball (14) (16); Hill (152a)
Lomatiol	Plant	−0.184	30	1 by 1	Ball (14); Hill (152a)
Riboflavin	Animal and vegetable	−0.208	30	1 by 1	Michaelis, Schubert and Smythe (232)
Echinochrome	Animal	−0.221	30	2 by 2 (?)	Cannan (57)

containing only R is titrated with an oxidant the magnitude of k will determine how much of S can be formed *in maximo* during the titration. If k is very small the two steps will greatly overlap. If this constant is very large, at the beginning of the oxidative titration, only S will be formed, and T will arise only after practically all R has been converted into S. In this case each of the two steps of oxidation can be treated separately, as there is practically no overlapping. Accordingly k can be estimated from the curve obtained by experiments. From the titration curve E_1 (the difference of potential at 50 per cent of the titration

and that at 25 per cent), the index potential is determined by graphic interpolation. E_m (the normal potential of the system corresponds to Clark's E'_0) is always the potential at 50 per cent of titration; E_2 is the potential at 75 per cent of titration. In general

$$E_m - E_1 = E_2 - E_m = \frac{RT}{F} \ln k.$$

The following oxidation-reduction systems naturally occurring in living cells have been found to transfer electrons one by one: riboflavin and phosphoriboflavin protein, pyocyanine, chlororaphine, hallochrome, toxoflavin, pthiocol, lapachol, and lomatiol. The two-step oxidation of pthiocol in alkaline solutions was shown by Hill (152); that of lapacol was demonstrated by Ball (16) and Hill (152a). It is quite likely that other systems (for example lawsone, juglone, which are naphthoquinone derivatives) present in biological fluids oxidize step by step. This opinion is supported by Hill's (152a) recent paper. As will be seen later, similar two-step oxidation has been found in pyridine-nucleotides and possibly thiamine. The case of phosphoriboflavin-protein is interesting because it seems that radical formation is increased when it combines with diphosphopyridine-nucleotide ((Haas) (144)).

Sluggish Oxidation-Reduction Systems. Sluggish oxidation-reduction systems are, as a rule, non-autoxidizable, i.e., they are not oxidized by atmospheric oxygen at a measurable rate and within pH values found in physiologic conditions. They are easily oxidized by electromotively active substances, and easily reduced by a number of oxidizable substances. Although thermodynamically reversible, they are electromotively sluggish and their potentials cannot be satisfactorily measured but with the aid of electroactive mediators. The potentials can then be measured potentiometrically, colorimetrically or spectrophotometrically, provided the sluggish system or the electroactive mediator can be determined by those methods. In effect, if we assume that thermodynamic equilibrium is established between the sluggish system *sl* ($(Ox)_{sl}^{+++} + e \rightleftharpoons (Red)_{sl}^{++}$) and the electroactive system *el* ($(Ox)_{el}^{+++} + e \rightleftharpoons (Red)_{el}^{++}$), the equilibrium constant K for the reaction is:

$$K = \frac{(Red_{sl})(Ox_{el})}{(Ox_{sl})(Red_{el})}$$

Since in the equilibrium mixture the potentials must be the same for both substances:

$$E'_{0sl} - E'_{0el} = \frac{RT}{F} \ln K; \qquad E'_{0sl} = E'_{0el} - \frac{RT}{F} \ln K.$$

This electroactive sluggishness was, before its recognition, a source of confusion and the origin of numerous conflicting statements about their reversibility.

Thiols. In 1921 Hopkins succeeded in isolating a sulphydril compound, first from yeast, then from all tissues, to which he gave the name glutathione (glutamyl-cysteinyl-glycin) (Hopkins (157), Kendall (180)). This discovery produced intense interest because it was thought at the time that an oxidation catalyst had been isolated, since the system could be easily reduced by tissues and, it was claimed, easily oxidized by atmospheric oxygen. The non-autoxidation of the thiol systems was demonstrated first for cysteine by Warburg and Sakuma (318); for glutathione by Meldrum and Dixon (222). Both cysteine and glutathione are easily oxidized by copper and hemochromogens (Krebs (188), Lyman and Barron (219)).

The failure of all the attempts made to measure the oxidation-reduction potential of thiols by direct potentiometric methods (Dixon and Quastel (86), Dixon (84), Michaelis and Flexner (231), Green (131), Ghosh and Ganguli (127)) is due partly to the sluggishness and partly to the formation of complex compounds between the electrode and the thiol (Barron, Flexner, and Michaelis (30)). Recently, measurements made under conditions described as thermodynamically reversible have been published, although the values found (E'_0 at pH 7.0 for the reaction 2 cysteine $\rightleftharpoons$ cystine + 2 H^+ + 2 e, +0.13 volt (Williams and Drissen (323)); −0.34 volt (Ghosh, Raychandri, and Ganguli (128); −0.23 volt (Fruton and Clarke (119))) differ too much among each other, as well as from those obtained from thermal data (−0.40 volt (Borsook, Ellis, and Huffman (45))) to be considered as due to experimental errors.

The potential of thiols ((S—S) + 2 e + 2 $H^+ \rightleftharpoons$ 2(SH)) at a given pH may be written:

$$E_h = E'_0 - \frac{RT}{nF} \ln \frac{(SH)^2}{(S - S)}$$

If the sum of the concentrations of (SH) and (S—S) is rewritten in the form of a degree of oxidation (x) of (SH):

$$E_h = E'_0 + \frac{RT}{nF} \ln 2\,SH\,\frac{(1 - x)^2}{x}$$

The potential observed is thus a function not only of the degree of oxidation but also of the concentration.

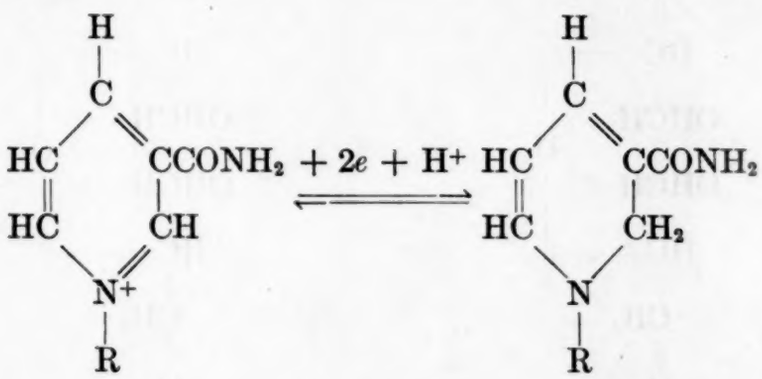
Ascorbic Acid. Another system of great reducing power is ascorbic acid (see review by King (181)), which, like glutathione, is present in biological fluids mostly in its reduced form. Non-autoxidizable in neutral solutions, it is easily oxidized by metallic catalysts, such as copper, and by hemochromogens (Barron, DeMeio, and Klemperer (29)). Protected in animal fluids from oxidation by glutathione and proteins (Barron, Barron, and Klemperer (28)) it is oxidized in certain vegetable fluids, the oxidation being due to the absence of protective power and the presence of oxidizing catalysts which are considered by some investigators as specific enzymes (see Tauber's review (287)). Some of these catalysts ("ascorbic acid oxidase") have an iron-porphyrin as active nucleus, some of them may resemble copper protein complexes (Stotz et al. (278)). In small concentrations, glutathione protects the oxidation of ascorbic acid by combining with copper present in the solute; in large concentrations, it prevents the oxidation of ascorbic acid (Borsook et al. (44)) by virtue of its more negative potential; under such conditions (ratio of glutathione: ascorbic acid, 100:1) glutathione may also reduce dehydroascorbic acid. Much has been written about the reversibility of ascorbic acid oxidation catalyzed by "ascorbic acid oxidase." The chemical reversibility of the system is conditioned exclusively by the hydrogen ion concentration, as shown in studies on the oxidation of ascorbic acid by copper, and its reduction by H_2S, the degree of reversibility depending on the degree of stability of the reversible oxidation product.

Characteristic proof of the sluggishness of this system is found in the contradictory statements about its thermodynamic reversibility, some investigators maintaining that the system is thermodynamically irreversible (Laki (201), Karrer, Schwarzenbach, and Schöpp (163), Green (132)), others maintaining that it is reversible (Georgescu (121), Borsook and Keighley (46), Wurmser and de Loureiro (330), Fruton (118)). By using the technique required for the determination of the potential of this group of compounds, that is, the addition of an electroactive mediator, Ball (14) restudied the subject and confirmed the reversibility of the system.

Pyridine-nucleotides. (Codehydrogenases, coenzymes, coferments.) (Myrback has written a review of diphosphopyridine-nucleotide (cozymase). See also reviews by Euler (94, 95) and Warburg (304).)

We owe our knowledge of the chemical constitution of pyridine nucleotides to the laboratories of Euler, Warburg, and Karrer. In 1934

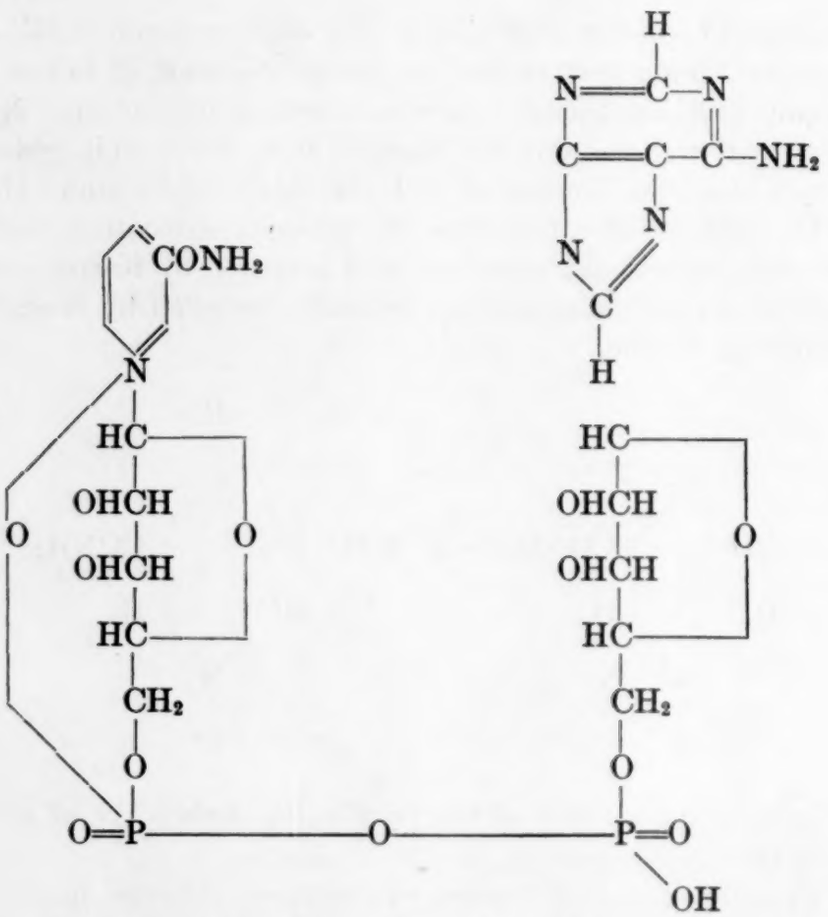
Warburg and Christian (308) found that their "coferment II" (one of the systems taking part in the enzymatic oxidation of hexose monophosphoric acid) contained a nicotinic acid amide group. Nicotinic acid amide is not reduced by $Na_2S_2O_4$; it is irreversibly reduced by Pt-H_2 to a piperidine compound with the uptake of six atoms of hydrogen. The addition of a radical to the nitrogen of nicotinic acid amide (for example, iodo-methyl-nicotinic acid prepared by Karrer and Warburg (165)) makes the compound reversibly reduced by $Na_2S_2O_4$ with the liberation of acid.



This is the active nucleus of the pyridinium derivatives of biological significance.

The oxidation-reduction process can be easily followed spectrophotometrically because the oxidized compound has (a characteristic property of these pyridinium derivatives) a strong absorption band around 2600 Å which on reduction diminishes, being replaced by another at 3400 Å, and because the reduced compound shows strong fluorescence under a white light (Warburg and Christian (308)).

The two pyridine nucleotides so far isolated, "cozymase" and "coenzyme II" contain besides the active nucleus, nicotinic acid amide, a purine, adenine, two pentoses, α-ribose, and phosphoric acids. The phosphoric acid is linked to ribose in the 5 position according to Euler and Karrer (101). "Cozymase" contains two phosphoric acid groups being a diphosphopyridine nucleotide. Its structural formula—tentatively suggested by Euler and Schlenck (102)—is



Euler and Schlenk (102) and Ochoa (243) have described simple methods for the preparation of diphosphopyridine nucleotide from yeast and muscle.

Coenzyme II is a triphosphopyridine nucleotide. Warburg, Christian, and Griese (312) prepared it from horse red cells. It can now be prepared from diphosphopyridine nucleotide by phosphorylation with $POCl_3$ (Schlenk (263)) or by enzymic phosphorylation (Euler and Adler (97), Vestin (298)).

Pyridine nucleotides are two-step oxidation systems; thus the methyl iodide of nicotinic acid amide when reduced with $Na_2S_2O_4$ in slightly alkaline solutions (pH 8.0) gives an intermediate compound, a free radical, of orange-yellow color (Karrer and Benz (161)). An intermediate reduction compound has also been found in the reduction of diphosphopyridine nucleotide (Adler, Hellström and Euler (2)) which is considered to be "monohydrocozymase" by Hellström (151).

In the reduced state and at physiological pH values none of these compounds is oxidized at a measurable rate by atmospheric oxygen. They are oxidized with great speed by phosphoriboflavin protein (Haas

(144)) and other electroactive systems of suitable potential (methylene blue, possibly hemochromogens). In the cell it seems that the reduced phosphopyridine nucleotides are oxidized by an enzyme discovered independently by Dewan and Green (77) and Adler, Euler, and Hellström (1). The enzyme has been found in animal tissues, yeast, and bacteria (Green and Dewan (137)). Like cytochrome oxidase it strongly adheres to the solid structures of the cell; the enzyme seems to contain no flavoprotein; its chemical constitution is still unknown.

The electroactive sluggishness of pyridine nucleotides is demonstrated by the failure of the numerous attempts made to determine by direct potentiometric titration the potentials of synthetic nicotinic acid amide compounds (Karrer and co-workers (162)) and of diphosphopyridine nucleotide (Hellström (151)). The potentials of these systems may be measured electrometrically by the aid of electroactive systems or by equilibration with other reversible systems. Use of the first method may necessitate the preparation of more stable oxidation-reduction dyes of quite negative potentials, because these systems when reduced have, as a rule, strong reducing power. (Thus the orthoderivatives of dihydronicotinic acid, amide: -n-methyl-o-n-ethyl-o and n-propyl-o-n-butyl-o- gave E_h values around -0.48 volt at pH 9, which would give roughly an E'_0 of about -0.4 volt.) Use of the second method has been made by Clark (64) for calculating the potentials of diphosphopyridine nucleotide (combined with the activating protein for lactic acid oxidation?) from Euler et al. (100) measurements of the equilibrium constant k for the reaction

$$\text{Pyruvate} + \text{D.P.N.} \rightleftharpoons \text{Lactate} + \text{D.P.NH}_2$$

At pH 9 (25°C.) the E'_0 of diphosphopyridine-nucleotide would be -0.37 volt.[3] It seems therefore probable that all these pyridine-nucleotides may have quite negative potentials, their values changing slightly on changing the nature of the group attached to the quaternary N of nicotinic acid amide (Karrer, Schwarzenbach, Benz and Sohmssen (162), Karrer and Stare (164), Schlenk, Hellström, and Euler (264)).

As iron-porphyrins combine reversibly with nitrogenous substances—proteins among them—so pyridine nucleotide (D.P.N.) and its reduc-

[3] Schlenk, Hellström, and Euler (264) have made similar calculations. Unfortunately they chose for these calculations E_0 values for ethyl alcohol $\rightleftharpoons$ acetaldehyde which were given by Wurmser and Filitti-Wurmser (327) only as tentative values, and E_0 values for malic acid $\rightleftharpoons$ oxaloacetic acid (Laki (203)) which were obtained only at one concentration of reactants. The E'_0 values for diphosphopyridine nucleotides given by them are about 32 m.v. more negative than those calculated by Clark.

tion product ($D.P.NH_2$) combine reversibly with proteins (Negelein and Gerischer (240), Negelein and Wulff (242)):

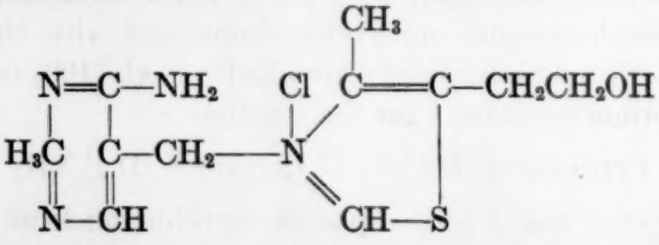
$$D.P.N. + \text{protein} \rightleftarrows P.N. - \text{protein} \quad (1)$$

$$D.P.NH_2 + \text{protein} \rightleftarrows D.P.NH_2 - \text{protein} \quad (2)$$

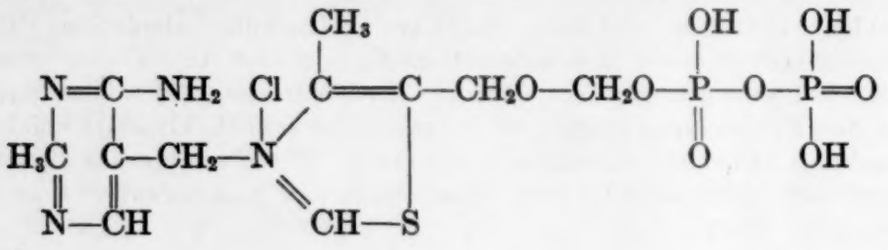
When diphosphopyridine nucleotide combines with the protein for the oxidation of ethyl alcohol, the equilibrium constants of reactions (1) and (2) have different values: $k = 9 \times 10^{-5}$ for reaction (1); 3×10^{-5} for reaction (2) (Negelein and Wulff (242)). When triphosphopyridine-nucleotide combines with the protein for the oxidation of hexosemonophosphate, the equilibrium constants for reactions (1) and (2) are the same: $k = 1 \times 10^{-5}$ (Negelein and Haas (241)).

Will the potentials of pyridine-nucleotides, like those of iron-porphyrins, change on combining reversibly with the proteins? Will in such a case Clark's conception of the relation of potential to the equilibrium constants of the oxidized and reduced system be applicable? These are problems for future research.

Thiamin (*vitamin* B_1), a thiazol derivative containing a primary alcohol and a quaternary pyrimidine ring, the structural formula of which was elucidated by Williams and his co-workers (324) contains also a quaternary nitrogen atom in a position comparable to its position in phosphopyridine-nucleotides:



Thiamin on phosphorylation takes up one or two phosphoric acid groups; thus is formed diphosphothiamin, "cocarboxylase," the constitution of which was established by Lohman and Schuster (217). The phosphorylation is performed enzymatically by phosphorylation enzymes (Tauber (286), Euler and Vestin (103), Lipschitz, Potter and Elvehjem (216)). Tauber (288) has recently succeeded in preparing diphosphothyamin synthetically from thiamin and pyrophosphoric acid.



Thiamin may be reduced with $Na_2S_2O_4$ or Pt-H_2 as Lipmann (215) has shown. Diphosphothiamin is also reduced with $Na_2S_2O_4$ (Barron and Lyman[4]). Both of these reduced substances are strong reducing agents and non-autoxidizable, i.e., not oxidized by atmospheric oxygen; they are, however, easily oxidized by the catalytic action of blood hemin and hemochromogens with an uptake of one atom of oxygen per mole of compound. These experiments and the close structural relation between thiamin and pyridine nucleotides made plausible the assumption that thiamin and diphosphothiamin are sluggish reversible oxidation-reduction systems. Unfortunately neither the reduced products nor the reoxidized compounds are physiologically active. It is quite possible that during the reduction process with $Na_2S_2O_4$ the thiamin molecule is split into pyrimidine and thiazol, as neutral sulfite solutions split thiamin at room temperature, a reaction which was discovered by Williams and was the starting point for his brilliant work on the synthesis of the vitamin.

Enzymatic-sluggish oxidation-reduction systems. When an indifferent electrode, gold or bright platinum, is immersed in an oxygen-free buffered solution containing a mixture of lactate and its oxidation product, pyruvate, no potential indicative of electron transfer is obtained at the electrode. The addition of α-hydroxyoxidase derived from gonococci gives to this system, lactate-pyruvate, a slight tendency to transfer electrons to the electrode; i.e., an erratic potential is obtained which drifts slowly towards negative values without attainment of equilibrium. But when an electromotively active system (for example, a dye) previously shown to be only partially reduced by this system, is added, there is obtained at the end of one to two hours a stable, reproducible potential. Although the potential is due directly to an electron transfer from the electroactive system, this system acts simply as a mediator, for its reduction has been brought about by the system lactate-enzyme-pyruvate. The values obtained may therefore be taken as representing the potential of this system. The potentials of these reversible systems thus require the mediation of two factors: the enzyme, which acts as a perfect catalyst, and the electroactive system, which is used to measure the potential of the system. These potentials may therefore be measured either electrometrically or colorimetrically.

The first of these systems to be discovered was the succinate-fumarate system. The reason for this early discovery can be seen in the wide use of methylene blue as an indicator of oxidation, which followed

[4] Unpublished experiments.

Ehrlich's pioneer work on "Das Sauerstoffbedürfnis des Organismus" published in 1883. It happens that the potential of this system lies around that of methylene blue. Wishart (325) discovered the reversibility; Quastel and Whetham (253) measured the equilibrium constant, and Borsook and Schott (47) demonstrated that the values for the free energy of this system, as obtained colorimetrically (Quastel and Whetham (253), Thunberg (294) or electrometrically, Lehmann (208), Borsook and Schott (47)) agreed remarkably well with those obtained with the help of the third law of thermodynamics.

Since then a number of enzymatic sluggish oxidation-reduction systems have been discovered covering a wide range of potentials, from the hydrogen potential (the system $HCO_2^- + H_2O \rightleftharpoons HCO_3^- + H_2$) studied by Woods (326) up to the methylene blue potential (the succinate $\rightleftharpoons$ fumarate system).

TABLE 4

Potentials of enzymatic sluggish oxidation-reduction systems; E'_0 values at pH 7.0

SYSTEM	E'_0	TEMP.	REFERENCE
	volts	*°C.*	
Succinate $\rightleftharpoons$ fumarate	0.000	38	Lehmann (208); Borsook and Schott (47)
d(−)Alanine $\rightleftharpoons$ ammonium pyruvate	−0.048	37	Wurmser and Filitti-Wurmser (329)
Malate $\rightleftharpoons$ oxaloacetate	−0.169	37	Laki (203)
Lactate $\rightleftharpoons$ pyruvate	−0.180	35	Barron and Hastings (33); Wurmser and Mayer-Reich (331)
Ethyl alcohol $\rightleftharpoons$ acetaldehyde	−0.190	30	Calculated from Adler's equilibrium measurements (3)
Isopropyl alcohol $\rightleftharpoons$ acetone	−0.251	30	Wurmser and Filitti-Wurmser (327)
β-Hydroxybutyrate $\rightleftharpoons$ aceto-acetate	−0.282	38	Green, Dewan and Leloir (138)
Xanthine $\rightleftharpoons$ uric acid	−0.361	30	Green (134); Filitti (106)
Hypoxanthine $\rightleftharpoons$ xanthine	−0.371	30	Ibid; ibid.
Formate $\rightleftharpoons$ $H_2 + CO_2$	−0.420	30	Woods (326)

The system ethyl alcohol $\rightleftharpoons$ acetaldehyde (important because it is to alcoholic fermentation what the system lactic acid $\rightleftharpoons$ pyruvic acid is to lactic acid fermentation) was studied by Lehman (209); as calculated from Lehmann's paper the E'_0 of this system at pH 7.0 is −0.063 volt; the value of −0.190 volt given in table 4 was obtained by calculation from the equilibrium measurements reported by Adler and Sreenivasaya (3). The equilibrium constant K for the reactions alcohol $\rightleftharpoons$ acetaldehyde and diphosphopyridine nucleotide (D.P.N.) $\rightleftharpoons$ dihydrodiphosphopyridine nucleotide (D.P.NH_2),

$$K = \frac{[\text{D.P.N.}]\,[CH_3CHOH]}{[\text{D.P.}NH_2]\,[CH_3COH]}$$

was determined at various pH values.

The potential of alcohol $\rightleftharpoons$ acetaldehyde is:

$$E'_{0_{\text{alcohol}}} = E'_{0_{\text{D.P.N.}}} - \frac{RT}{2F} \ln K.$$

(The E'_0 values of diphosphopyridine nucleotide given by Clark (64) were used.) The difference between this value and that obtained by Lehmann is too great to be attributed to experimental error. To check the validity of both values, those calculated by Clark for diphosphopyridine nucleotide and those calculated here for alcohol $\rightleftharpoons$ acetaldehyde, one can determine the free energy ΔF of this reaction with data where no enzyme is concerned. Parks and Huffman (246) have calculated the free energy for the reaction

$$\text{CH}_3\text{CH}_2\text{OH (gas)} = \text{CH}_3\text{CHO (gas)} + \text{H}_2$$
$$\Delta F^\circ = 11{,}400 - 4.0\ T \ln T + 9.96\ T^5$$
$$\Delta F_{298^\circ} = 7586 \text{ calories}$$

For the reaction in aqueous solution, to calculate the molal free energy change, the vapor pressure data for acetaldehyde given by Bushmakin and Kuchinskaya (55) (99 mm. Hg per 1 M acetaldehyde) and that of alcohol given by Dobson (87) (3.65 mm. Hg) were used. As a result:

$$\Delta F^0_{298} = 7586 + RT \ln \frac{99}{3.65} = 9540 \text{ calories } (E_0 = +0.230 \text{ volt})$$

ΔF_{298° as calculated from the E_0 value (+0.223 volt) is 10280 calories. The agreement is satisfactory. Moreover, it gives greater validity to Clark's calculations of the oxidation-reduction potential of diphosphopyridine nucleotide.

The reaction α-glycerophosphate $\rightleftharpoons$ glyceraldehyde phosphate is also reversible (Meyerhof and Kiessling (226)). The equilibrium constant for the reaction,

α-glycerophosphate + D.P.N. $\rightleftharpoons$

glyceraldehyde phosphate + D.P.NH$_2$,

has been measured at 30° by Euler et al. (99). The E'_0 at pH 7.0 for the reaction would be −0.180 volt.

Free Energies and Rates of Reactions. It seems that a number of biologists still stress the importance of the heat of reaction and consider

[5] The value of 9.96 for I (integration constant of the free energy equation) is the average obtained from Bancroft and George's (20) data on the equilibrium of this reaction.

it the criterion for determining the direction of a chemical reaction. In the latter point, however, they are incorrect, because the factor which determines that a given process will take place, or at least *may* take place spontaneously, is not the heat evolved in the process but rather the free energy evolved. If the reaction proceeds at constant pressure

$$\Delta F = \Delta H - T\Delta S.$$

The free energy decrease $-\Delta F$ of the reaction is equal to the heat of the reaction $-\Delta H$ only in case $\left(\frac{\delta(\Delta F)}{\delta T}\right)_p = 0$, where p = pressure. If the temperature coefficient of the free energy decrease of a reaction at constant pressure is negative, $-\Delta F > -\Delta H$; if it is positive, $-\Delta F < -\Delta H$. Thus a knowledge of ΔF is of great utility to the biologist.

If ΔF has a large negative value, the process involved may take place spontaneously at constant temperature and pressure; if ΔF is positive for a process, there must be an increase of free energy in the system and the process will take place only through the application of work; if ΔF is zero, a state of equilibrium exists and no further change is to be expected. Constant concentrations other than standard are here assumed.

From a knowledge of ΔF for the process at one atmosphere the equilibrium constant K for the reaction can be calculated by means of the thermodynamic relationship

$$\Delta F^\circ{}_T = -RT \ln K.$$

Neglect of these fundamental considerations has led to a number of misconceptions. From quite an extensive bibliography let us quote only two examples: Wieland (327) reported the oxidation of hydroquinone by palladium, a reaction which requires so large a quantity of energy "that the probability of the process occurring in the direction specified falls in the same category as the probability that a kettle of water will boil when placed on ice" (Clark (63)). The reaction as stated by Wieland and presented as example of his "hydrogen activation" theory was proved by Gillespie and Liu (130) not to take place. Szent-Györgyi (282) observed that paraphenylene diamine was rapidly oxidized by washed muscle tissue in the presence of oxygen; that oxidation did not occur when methylene blue instead of oxygen was used as "hydrogen acceptor." He then concluded: "*Bei der Oxydation des Paraphenylenediamins durch das Muskelgewebe findet also keine Wasserstoffaktivierung statt.*" If Szent Györgyi had been in possession of the

free energy data of these two reversible systems (E'_0 at pH 7.0, temp. 30°, of the p. phenylenediamine $\rightleftharpoons$ quinonediimine system, +0.381 volt (Fieser (105)); of methylene blue, +0.011 volt (Clark, Cohen and Gibbs (66))) he would not have been put to the necessity of postulating that the oxidation of p-phenylene diamine was not performed through hydrogen activation.

Very valuable work, that of determining the free energies of substances of biological importance, is now being done at the California Institute of Technology by Borsook, Huffman, Parks and co-workers.

While ΔF enables us to predict the direction and extent of a reaction, it can tell us nothing regarding the time necessary for the attainment of a condition of equilibrium. "At one time the equation Reaction velocity $= \frac{\text{Chemical affinity}}{\text{Chemical resistance}}$ was used in a qualitative and descriptive way, but it is rather misleading, because there is no correlation whatever between chemical affinity, as measured by the maximum work, and speed of reaction. Affinity depends solely upon initial and final states. Velocity of reaction, on the other hand, depends upon the nature of some specific reactive state in which the molecules become capable of rearrangement" (Hinshelwood (154)). This statement summarizes the position taken by students of chemical kinetics whenever an attempt was made to correlate free energy with speed of reaction. However, some voices of protest were heard from time to time. Witness Conant's work in 1926 (70) on "the apparent reduction potential" of reducing substances capable of an irreversible reduction; LaMer and Temple's work in 1929 who reported that the rate of oxidation of hyroquinone under the catalytic influence of manganous salts was a function of the available free energy of the system. In 1930 (Barron and Hoffman, 34) it was found that the catalytic power of reversible dyes on cellular respiration was conditioned by two factors: the oxidation-reduction potential of the dye, and the permeability of the cell membrane; the speed of this catalysis was correlated to (1) the speed at which the dye is reduced by the cell, and (2) the speed at which the leuco-dye is oxidized by atmospheric oxygen. Such a correlation between the free energy of the dyes (as determined by their potentials) and their influence on cell respiration was naturally received with caution. However, a study of the rate of oxidation by atmospheric oxygen of reduced electroactive dyes showed the same relation between the free energy and the rate of oxidation (Barron (25)). Since then, numerous papers have been published from different laboratories,

confirming these observations (Stotz and Hastings (279), Chow and Kammerling (61), Chow (60), Hammett (146), Laki (202), Dimroth (81), Dewan and Green (78)).

The theories of reactions recently developed by Eyring (104), Rice (257, 258), La Mer (204) and Gerschinowitz (126), which make explicit use of an activated complex, show that the rate of a reaction is determined by a free energy change which is usually not the same as the free energy for the total reaction. They have shown that the rate of a chemical reaction is determined by the concentration of the reacting systems in a certain fraction of the total phase space that is available to the systems, multiplied by the velocity with which the systems are passing through this region. The configuration of the reacting molecules which corresponds to this phase space may be called the active complex. They postulate (see Gerschinowitz for derivation of equation) that the specific rate constant (k_1) of these reactions depends on the free energies of the activated systems:

$$k_1 = \exp \frac{-\Delta F^*}{RT} \frac{kT}{h}$$

where ΔF^* is the free energy of formation of the activated state, and k and h the Boltzmann and Planck constants, respectively. In general, ΔF^* is not the same as ΔF, the free energy change for the total reaction. In order to have the free energy change in such reactions equal to the free energy of formation of the activated complex, it would be necessary that the energy of formation of the activated state be equal to the energy of the reaction. (Three such cases have been reported: LaMer and Temple (205), Dimroth (81), and Barron (25).)

The relation of free energies to rates of oxidation seem also to be controlled by what Shaffer (265, 266) calls "the equi-valence-change principle," a theory of great importance for its biological implications. One example given by him will illustrate the theory: Ce^{+++} ions in N H_2SO_4 ($Ce^{+++} + 1\ e \rightleftharpoons Ce^{++}$, $E_0 = +1.431$ volt, Baur and Glasson (39)) oxidize Tl^+ ions ($Tl^{+++} + 2\ e \rightleftharpoons Tl^+$, $E_0 = +1.206$, Sherrill and Haas (267)) very slowly, although both are electroactive oxidation-reduction systems and the potential of the oxidant (Ce^{+++}) is high enough to oxidize the reductant (Tl^+). If to such a system $MnSO_4$ is added (manganese ions may exchange electrons one by one or two at a time: $Mn^{++++} + 1\ e$ Mn^{+++}, $E_0 = +1.642$ volts; $Mn^{++++} + 2\ e \rightleftharpoons Mn^{++}$, $E_0 = +1.577$ volts, Grube and Huberich (142)), the oxidation of thallous ions by ceric ions will proceed rapidly. According to

Shaffer, the oxidation is slow because the oxidant, Ce^{+++}, is a one-electron system, while the reductant, Tl^{+}, is a two-electron system; such a reaction would require a trimolecular collision, an event of such rarity that, if in addition to a molecular encounter considerable activation is required, the velocity of reaction will be negligibly small. Manganese acts in this reaction as catalyst because it can lose and accept one or two electrons and hence can replace a trimolecular collision with a series of bimolecular collisions. For the same reason a system capable of semiquinone formation could also act as catalyst. Shaffer has indeed found that various dyes which oxidize in two steps (pyocyanine, other phenazines) may act as catalysts in the above mentioned oxidation. Shaffer's theory has found strong support in Michaelis' discovery of the two-step oxidation systems, systems which are found in cells and whose rôle as catalysts has been amply proved (pyridine-nucleotides, alloxazins, phenazines, quinones). Michaelis has embodied Shaffer's ideas in what he calls the "principle of compulsory univalent oxidation." According to him any oxidation can proceed only in successive univalent steps. The lack of reactivity toward oxidation in general would be due to the fact that usually the formation of the intermediate radical requires a step-up in free energy. The free radical may be taken as the equivalent to the activated state of the molecule. So far, experimental findings favor these conceptions. They resemble Brönsted's theory for acid-base catalysis (52) as enlarged by Pedersen (249); for, according to them, the reaction between an acid catalyst HA and a substrate S,

$$HA + S \rightarrow \text{Products} + HA,$$

takes place in the following steps:

$$HA + S \rightleftharpoons SH^{+} + A^{-}$$
$$SH^{+} + A^{-} \rightarrow \text{Products} + HA.$$

The Rôle of Oxidation-Reduction Systems in Cellular Respiration. The three groups of reversible oxidation-reduction systems which are found in cells act as components of the oxidizing enzyme systems. As a rule the sluggish systems act as oxidizing catalysts of the activated substrate; the electroactive systems as the electron mediators with molecular oxygen. For this reason it will be well to start by discussing the rôle of the sluggish systems, and end with that of electroactive systems.

Sluggish oxidation-reduction systems. The discovery by Warburg and his school that pyridine nucleotides are *actual* components of

oxidation enzymes has, indeed, been of first importance to the understanding of the mechanism of cellular respiration, for this group of substances can be taken as the best example of sluggish reversible systems.

The function of diphosphopyridine nucleotide (Euler's cozymase, Warburg's coenzyme II) in biological oxidations has been established in a number of contributions by Green and his co-workers, and Euler and his co-workers. A number of carbohydrate derivatives activated by their specific activating proteins have been shown to be oxidized, in isolated enzyme systems, with diphosphopyridine nucleotide as an electron mediator: alcohol, lactic and malic acids, formic acid, glutamic acid, triose phosphate, citric acid, glucose (Euler and associates, Green and Dewan (136), Dewan and Green (78), Meyerhof and Ohlmeyer (225), Anderson (6)). Diphosphopyridine nucleotide has an important function as mediator in the series of oxidation-reductions going on during carbohydrate fermentation. The electron transfer from the reduced nucleotide seems to be mediated mainly through a pyridine nucleotide oxidase, an enzyme discovered by Green and by Euler. The orientation and speed of these reactions will be governed by thermodynamic relationships, concentration of reactants, oxygen tension, electrolyte concentration, and other factors still unknown.

The rôle of triphosphopyridine nucleotide in cellular respiration is not yet known. It is indeed of extreme interest that this nucleotide which can mediate the oxidation of hexose-monophosphate when activated by the protein purified by Negelein and Haas (241), can also act as mediator for the further oxidation of phosphohexonic acid when in the presence of another protein (Warburg and Christian (309)). We are once more in the presence of a series of oxidations, the oxidation of glucose being performed by steps. Is the potential of triphosphopyridine nucleotide altered by union with the specific activating proteins, thus allowing a gradual oxidation of the split products of the original hexose-monophosphate? Further research is needed to answer this question.

Other oxidations where triphosphopyridine nucleotide acts as an electron mediator are the oxidation of glucose (Harrison (148)) and of glutamic acid (Euler, Adler and Eriksen (98)). Das (75) reports that both pyridine nucleotides may act as mediators of the oxidation of glucose.

It is remarkable that the respiratory function of the sluggish system, glutathione, discovered as long ago as 1921 by Hopkins, the chemical constitution of which was established in 1929, and its synthesis effected in 1935 (Harrington and Mead (147)), is still unsolved. Can gluta-

thione act as an electron mediator between an activated substrate and an electroactive catalyst? Its oxidation-reduction potential seems to be about that of diphosphopyridine-nucleotide; Hopkins and Elliott showed in 1931 (158) that the system could be reversibly oxidized and reduced by the liver of well-fed animals. Nevertheless, no experimental proof of the catalytic power of glutathione in biological oxidations has yet been offered. The same may be said of ascorbic acid. Both sluggish systems seem to act as mediators of the processes of synthesis.

Further work in the field of sluggish oxidation-reduction systems will doubtless bring about the discovery of other substances acting as mediators between the activated substrate and the electroactive catalyst.

A point of special importance, not yet studied, is whether the combination of pyridine nucleotides with the activating protein—a combination the reversible nature of which has been reported by the associates of Warburg—alters the oxidation-reduction potential of the system. If we assume that Clark's treatment of the effect of nitrogenous compounds on the potentials of hemin applies generally to systems combining reversibly, we may predict that the potentials of pyridine nucleotides will change on combination with proteins, i.e., the potential would become more positive if the reduced pyridine nucleotide associated more strongly with the protein, more negative if the oxidized component associated more strongly, or remain unaltered if the affinity for reductant and oxidant is the same. This study is of significance because it may determine whether the union of the activating protein to the pyridine nucleotide is an ordinary chemical combination or is only similar to those postulated in adsorption phenomena, leaving, in the latter case, the free energy of the system unaltered.

Enzymatic sluggish oxidation-reduction systems. It has been shown that although these systems are thermodynamically reversible, the rate of electron transfer does not become appreciable until an electroactive catalyst is added, which acts as an electron mediator. Given, therefore, a number of enzymatic oxidation-reduction systems of graded potentials and a number of electroactive systems of potentials lying between those of the sluggish systems, we may build a series of oxidation-reduction systems where the electron transfer may proceed from the system of more negative potential to the system of more positive potential. The enzymatic-sluggish oxidation-reduction systems may therefore act like the pyridine-nucleotides, i.e., as catalysts. Szent-Györgyi and his collaborators (284) have presented evidence of

the existence of such a series in their theory of C_4 dicarboxylic acids as catalysts for cellular respiration. According to them, triosephosphates are oxidized by oxalo-acetic acid; the malic acid formed in the oxidation is oxidized by fumaric acid; the succinic acid thus formed is oxidized by the cytochrome system. It is reasonable to believe that when enzymatic-sluggish reversible systems like those of Szent-Györgyi's system do exist cellular respiration as a whole may proceed through this series. Stare (273), Stare and Baumann (271) Boyland and Boyland (48), Greville (141), Leloir and Dixon (211), and others have offered evidence in favor of Szent-Györgyi's catalysts. (The papers from Szent-Györgyi's laboratory can be found discussed as a whole in Szent-Györgyi's book, *Studies on biological oxidation and some of its catalysts.*) The citric acid cycle postulated by Krebs and Johnson (191) would involve other reversible-sluggish systems, isocitric $\rightleftharpoons$ cisaconitic acid, a reversible reaction, according to Martius and Knoop (221), and the reaction, citric acid $\rightleftharpoons$ cisaconitic acid, also reversible, according to Breusch (51). Whether carbohydrate oxidation goes through Szent-Györgyi's cycle, Krebs' cycle, or through direct oxidation of pyruvic acid will depend not only on the presence or absence of the specific activating proteins and the electroactive electron mediators but also on all the factors which determine the orientation of these reversible reactions.

Electroactive oxidation-reduction systems. The property of electroactive systems of being autoxidizable, united to that of ready electron transfer with other oxidation-reduction systems, explains the function of this group. They may act as electron mediators between the activated substrate and molecular oxygen; between a sluggish mediator system (pyridine nucleotides, cytochrome *c*) and molecular oxygen; between enzymatic-sluggish oxidation-reduction systems (Borsook's coupled reactions or Szent-Györgyi's C_4 dicarboxylic acids); and finally, between one another (Theorell (290)), the system of more positive potential oxidizing that of more negative potential, in a series.

The catalytic power of an electroactive system in cellular respiration was demonstrated in 1928 (Harrop and Barron (149)). It was found at that time that the addition of methylene blue brought about a considerable increase in the respiration of mammalian erythrocytes, which was accompanied with an increased disappearance of glucose and diminution of lactic acid formation (Barron and Harrop (31)). The catalytic effect is insensitive to HCN (Harrop and Barron (149)) and to CO (Gerard (124)). It was found in all tissues which produced lactic acid in aerobic conditions: leucocytes, tumour tissues, echinoderm

eggs (Barron (24)), muscle (Gerard (123)), fish erythrocytes (Chapheau (59)), nerve (Gerard (122)), retina (Fleischmann and Kann (110)). Previous to these experiments *with living cells*, Meyerhof had discovered in 1917 (223) that addition of methylene blue to acetone-treated bacteria produced a rapid oxygen consumption; Fleisch (109) had shown in 1924 that the oxidation of succinic acid by an enzyme preparation from muscle, when inhibited by HCN, was restored on addition of methylene blue. This catalysis was explained as the result of direct electron transfer between the oxidizable substrate and dye, and between dye and atmospheric oxygen. Wendel (320) and Warburg, Kubowitz, and Christian (314), who discovered that the product of lactic acid oxidation was pyruvic acid, postulated a theory by which the dyes oxidize hemoglobin (in experiments with red cells) and that the methemoglobin thus formed acted as an oxidant of lactic acid. Using red cells and as catalysts dyes of a potential unable to oxidize hemoglobin, DeMeio et al. (76) showed that the catalytic effect of methylene blue could be taken as a model to explain the function of electroactive systems:

$$\text{Lactate}^- + \text{M.B.} \rightarrow \text{Pyruvate} + \text{L.M.B.} + 2\,\text{H}^+ \quad (1)$$

$$\text{L.M.B.} + \text{O}_2 \rightarrow \text{M.B.} + 2\,\text{O}^- \quad (2)$$

$$2\,\text{H}^+ + 2\,\text{O}^- \rightarrow \text{H}_2\text{O}_2 \quad (3)$$

In cells possessing catalase, H_2O_2 is immediately decomposed.

a. Iron-porphyrins. The systems containing iron-porphyrin as the active nucleus are by far the most important. In fact this nucleus is present in the cytochromes of Keilin, in Warburg's "Sauerstoffübertragende Ferment der Atmung," in most peroxidases (Kuhn, Hand, and Florkin (196), Keilin and Mann (175)), in catalase (Zeile and Hellström (336), Stern (276), Sumner and Dounce (281)). Furthermore the iron-porphyrin nucleus possesses the interesting property of forming three sorts of compounds each of them playing a fundamental part in cellular respiration. Iron-porphyrin may unite with a protein and thus combine reversibly with molecular oxygen without electron exchange (hemoglobin, myoglobin, possibly other heme-protein compounds not yet isolated). The loading and unloading of oxygen by these oxygen store pigments will depend on the value of the equilibrium constant (Barcroft (23), Millikan (234)); iron-porphyrin may combine with a protein forming a sluggish oxidation-reduction system, non-autoxidizable (cytochrome *c* and possibly other cytochromes); iron-porphyrin may combine with nitrogenous compounds giving autoxidiz-

able electroactive systems (hemochromogens, Keilin's "cytochrome oxidase"). Thus iron-porphyrin compounds may act as mediators between activated substrates and electroactive systems, and as final mediators with molecular oxygen.

The function of cytochromes has been recently discussed by Keilin (169) and Shibata (268). Cytochrome *c*, a sluggish reversible system, seems to act, like the pyridine-nucleotides, as electron mediator between the activated substrate and the autoxidizable catalyst; it acts in this way in the oxidation of succinic acid. When the three cytochromes are present in cells they may all take part in the oxidation-reduction series:

1. $2\ Fe^{++}$ cyt $b + 2\ Fe^{+++}$ cyt $c \rightarrow 2\ Fe^{++}$ cyt $c + 2\ Fe^{+++}$ cyt b; (E'_0 cyt b, -0.04 volt)
2. $2\ Fe^{++}$ cyt $c + 2\ Fe^{+++}$ cyt $a \rightarrow 2\ Fe^{++}$ cyt $a + 2\ Fe^{+++}$ cyt c; (E'_0 cyt c, $+\ 0.255$ volt)
3. $2\ Fe^{++}$ cyt $a + O_2 \rightarrow Fe^{+++}$ cyt $a + 2\ O^-$ (E'_0 cyt a, $+0.29$ volt)

Equation 3 is hypothetical. Whether cytochrome *a* is autoxidizable or not is not known with certainty, though Keilin and Hartree (174) have observed that part at least of cytochrome *a* is autoxidizable.

Whether Keilin's "cytochrome oxidase" or Warburg's "Sauerstoff-übertragende Ferment" is cytochrome *a* or a hemochromogen of higher potential is not known. The property of iron-porphyrins of combining reversibly with nitrogenous compounds to give oxidation-reduction systems of varied potential has made of them the almost universal mediators of electrons in the last steps of cellular respiration.

As hemochromogens combine reversibly with HCN and CO, the inhibition in oxidation produced by these two compounds was taken as an indication of oxidation with hemes as catalysts. However, Sato and Tamiya (262) have shown the existence of cells which although possessing some of these cytochromes had their respiration inhibited by HCN and not by CO, and of cells whose respiration was inhibited by neither. Finally, there are oxidations catalyzed by hemin, as the oxidation of unsaturated fatty acids, where HCN has no inhibiting effect (Barron and Lyman (36)).

Alloxazins. As soon as Warburg discovered his "Yellow Ferment" a series of papers appeared from different laboratories (see Wagner-Jauregg's review (300) and Euler's review (94)) maintaining that this system acted as electroactive mediator between molecular oxygen and

the next member in the series of oxidation-reduction systems (generally a pyridine nucleotide). It was even called "Methylenblau der Zelle." As an electroactive system, indeed, it may act as such under conditions where no other electroactive system of higher potential is present. But even in such cases, when there are cytochromes present, and the oxygen tension is lowered to that presumably existing in the cells, phosphoriboflavin protein is reoxidized through the cytochrome system (Theorell (290)). The function of alloxazins in cellular respiration remained unknown until 1938 when the two important contributions from Warburg's laboratory already referred to were published (one by Warburg and Christian (311) on d-amino acid oxidase; the other by Ball (18) on xanthine oxidase). The prosthetic group of these two oxidizing enzymes is made up of alloxazin-adenine-dinucleotides; when combined with the specific activating proteins these alloxazins oxidize the oxidizable substrate, the reduced alloxazin being reoxidized by atmospheric oxygen. Alloxazin dinucleotide from d-amino acid oxidase on combining with the protein of the yellow ferment actively oxidizes dihydrodiphosphopyridine nucleotide (reduced cozymase). The prosthetic group of Green's "coenzyme factor" (pyridine nucleotide oxidase) might be an alloxazin dinucleotide. Alloxazin mononucleotide seems to have no action as oxidation catalyst. Phosphoriboflavin protein acts as an electron mediator for reduced pyridine nucleotides (Haas (144)). Electron transfer from the reduced flavoprotein to fumaric acid (activated by the proper protein) has been shown by Laki (202); and Banga (21) has shown that it may act as electron mediator between the reactions, malic acid and fumaric acid. Thus alloxazins seem to act in highly organized cells as electron mediators between sluggish oxidation-reduction systems. (Its rôle in glycolysis is a consequence of this assumption.) As an electroactive system, it may act as the final mediator of electrons in the series, if 1, no electroactive systems of higher potential are present, or if 2, such systems have been rendered inactive. Examples of the first class are found in the respiration of a number of bacteria (Warburg and Christian (306), Fujita and Kodama (120)). Examples of the second class are provided by the residual respiration of mammalian tissues after addition of HCN (Dixon and Elliot (85)). It must be recalled that, in general, bacteria devoid of iron-porphyrin compounds live by fermentation, anaerobically, the flavoprotein acting as electron mediator; in the presence of oxygen, the flavin acts as catalyst for oxidations, an oxygen uptake ensues and

H_2O_2 starts piling up (because of the absence of catalase and absence of coupled oxidation where H_2O_2 may act as oxidant). As Warburg and Christian (307) remarked, this is an unphysiologic process.

Other electroactive systems. (See table 3.) After the discovery of the catalytic action of methylene blue on cellular respiration, a number of papers appeared in the literature (Friedheim (112–116) describing the catalytic effect of electroactive systems normally present in cells. As these experiments were performed with cells of very low respiration (generally mammalian red cells) it must be said that the rôle of these pigments in cellular respiration is still unknown. *B. pyocyaneus* contains cytochromes besides pyocyanine, and its respiration is inhibited by HCN (Yamagutchi (334)). They may act either as the last step towards oxygen or as mediators of coupled oxidations (Michaelis and Smythe (233)).

Kisch and his co-workers (182–184) have devoted a number of papers to the study of the catalytic effect of oxidation-reduction systems of quite positive potential (quinones, pyrocatechol, adrenaline). According to the authors they act as good catalysts for oxidative deamination. Hodel (155) reported on the effect of adrenaline on cellular respiration. This effect may be due to adenochrome formation which according to Green and Richter (139) is an oxidation product of adrenaline which acts itself as a reversible system. Blasschko, Richter, and Schlossman (41) speak of an HCN-insensitive system in tissues which oxidizes adrenaline to adrenochrome. Whether these quinones, of which there are a number of living systems, act as normal catalysts for biological oxidations is not known. Their rôle as catalysts in plant respiration has been suggested by Palladin (245) and Oparin (244). Amino acids are oxidized in tissue extracts (Bernheim and Bernheim (40), Krebs (188, 189), Keilin and Hartree (170)) by two groups of oxidizing enzymes: one, extractable, HCN-insensitive, oxidizes d-amino acids; the other, non-extractable, HCN-sensitive, oxidizes l-amino acids. The components of the second enzyme are not yet known.

Activating Proteins (Dehydrogenase; Dehydrase, Apodehydrase, Zwischenferment). Gonococci suspended in 0.1 M NaF have the ability to oxidize, in the presence of oxygen, lactic acid to pyruvic acid (Barron and Hastings (32)). No other oxidizing enzyme is present in this suspension. On heating the suspension for 2 hours at 52°, the suspension was unable to oxidize lactate; the oxidation started as soon as an electroactive oxidation-reduction system of suitable potential was added. On heating to 70°, the oxidation did not proceed even on addition of the

electroactive system. The oxidation of lactic acid to pyruvic acid required thus the action of two factors equally important: one concerned with the activation of the lactate molecule; the other concerned with the oxidation of the activated lactate. The activation of the lactate molecule occurs at the surface of the activating enzymes, a protein, as only the lactate adsorbed by the enzyme can be oxidized.

Wieland (see his book (321)) and Thunberg (see his latest review (295)) deserve full credit for having been the pioneers for the discovery of the activating proteins, the isolation of some of which has been brilliantly performed in Warburg's laboratory; Negelein and Gerischer (240) prepared from yeast a protein (Zwischenferment) which specifically activated hexosemonophosphate; Negelein and Wulff (242) isolated from yeast a protein which specifically activated the reversible reaction alcohol $\rightleftharpoons$ acetaldehyde. (This is a clear example of the need for dropping names suceptible of misconception: this protein is called by Euler and his school "alcohol dehydrogenase," while Negelein calls it acetaldehyde "reductase." The protein may act either as "dehydrogenase" or "reductase" on altering the conditions which determine the value of the equilibrium constant.) Warburg and Christian (309) isolated a protein which activates the further oxidation of phosphohexonic acid. The activating protein of d-amino acid oxidase was isolated by Warburg and Christian (311). Ball (18) is working on the isolation of the activating protein of xanthine oxidase.

The specificity of the activating proteins has been satisfactorily demonstrated. These proteins may possess a species specificity, or a group specificity. Warburg's protein for the oxidation of hexose monophosphate seems to be the clearest example of the first group. Bernheim's extensive studies on the enzymes concerned with the oxidation of amino acids provide examples of group specificity. The enzymes obtained from milk, one able to oxidize purines ("xanthine oxidase"), the other able to oxidize aldehydes ("aldehyde oxidase") show less specificity. Considered by Wieland and Macrae (322) as two different enzymes, they have been shown by Booth (42) to be one enzyme. Here we have an enzyme activating purines (hypoxanthine, xanthine, and six other purines) and aldehydes.

Whether the protein is only a "colloidal carrier" of the pyridine-nucleotides and systems of similar oxidation-reduction properties, as Warburg and his school maintain, or whether it acts by activating the oxidizable molecule, i.e., altering the electronic configuration of the substrate in such a manner that it becomes ready to exchange electrons

with the pyridine nucleotides and systems analogous to them, is not yet known. Further work in this direction is necessary. Of special interest is the discovery of Hopkins and Morgan (159) that the activating protein for the oxidation of succinic acid loses its activity on treatment with oxidized glutathione, the activity being restored on further treatment with reduced glutathione. Similar findings have been reported by Rapkine (255) with triosephosphate oxidase. It would seem that an SH group in the protein is essential for its activity. (See Hellerman's review (150) on "Reversible inactivations of hydrolytic enzymes," where he deals with similar phenomena.) Wagner-Jauregg and Möller (299) have also reported that glutathione activates the activating protein of alcohol oxidase. Does the hypothetical system, 2 Protein SH $\rightleftharpoons$ Protein S-S-Protein + $2H^+$, act in the same way as the pyridine nucleotides?

Metallo-protein Complexes. When the oxidation-reduction potential of the oxidizable substrate approaches that of the oxygen potential the oxidation enzymes become less complicated. The oxidizing catalyst, generally a heavy metal, combines more firmly with the protein giving metallo-protein complexes. *Peroxidase* seems to be an iron-porphyrin-protein complex (Kuhn, Hand, and Florkin (196), Keilin and Mann (175)). *Catalase* has also been considered an iron-porphyrin-protein complex (Stern (277), Sumner and Dounce (281), Keilin and Hartree (171)). However, Agner reports (4) that catalase preparations of higher purity than those of Sumner's "crystalline catalase" contain copper (0.02–0.03 per cent) besides iron (0.085 per cent). Whether catalase contains two metallo-protein complexes whereby the ferrous complex formed on the decomposition of H_2O_2 is oxidized by the cupric complex, and the cuprous complex thus formed oxidized by molecular oxygen is not yet known. *Polyphenol oxidase* is a copper-protein complex containing 0.165 per cent copper (Kubowitz (194), Keilin and Mann (173)). *Uricase*, the enzyme which oxidizes uric acid to allantoin, seems to be an iron-protein complex (Davidson (75a)). In all these enzymes oxidation is performed by electron transfer between the metallo-complex (the -ic form) and the oxidizable substance and between the metallo-complex (the -ous form) and molecular oxygen. Further research in this group of enzymes may discover the existence of other metallo-protein complexes acting as catalysts in the oxidation of those substances of highly positive potentials.

The Oxidation-Reduction Series in Cellular Respiration. It has been shown that the cell possesses three kinds of reversible oxidation-reduc-

tion systems: the electromotively active systems, the sluggish systems, and the enzymatic-sluggish systems. As a rule, only the electromotively active systems transfer electrons to oxygen; these systems also transfer electrons between the sluggish systems. All of them, however, by virtue of their reversibility *may* take part in cellular respiration by transferring electrons from foodstuffs towards oxygen, their rôle being that of electron mediators or catalysts. Thus an oxidation-reduction series is established. Free energy relationships of necessity, will decide whether system *A may be* oxidized by system *B*. They may also determine the rate of reaction if we accept the postulate that the specific rate constant, k_1, of these reactions depends on the free energies of the activated systems (Gershinowitz (126)). The relation of free energy to rate of oxidation may also be conditioned according to Shaffer's equivalence principle and Michaelis' principle of compulsory univalent oxidation.

Activated foodstuffs seem to be oxidized in the cell in such a way that the release of energy of oxidation is performed through a series of reversible oxidation-reduction systems. Energy is thus released by gradual steps, the number of steps being determined by the number of systems making up the series.

The concept of the oxidation-reduction series explains the great number of coupled oxidations going on not only during respiration but also during fermentation (Quastel and Wooldridge (254), Borsook (43), Green, Stickland, and Tarr (140), Dewan and Green (78), Needham and Pillai (239), Meyerhof (224)). From the extensive literature, two examples may be taken to illustrate the mechanism of such a series. Borsook was the first to demonstrate that formate and pyruvate, in the presence of their respective activating enzymes, do not react; the addition of an electroactive catalyst of a potential value between that of the other two systems produced the oxidation of formate and reduction of pyruvate. There is in this case the following oxidation-reduction series:

$\text{Pyruvate}^- + 2\,\text{H}^+ + 2\,e \rightarrow \text{Lactate} -$	E'_0 (pH 7.0)	$-$ 0.180 v.
Methylene violet $+ 2\,\text{H}^+ + 2\,e \rightarrow$ Leuco methylene violet	E'_0 "	$-$ 0.260
$\text{Formate}^- + \text{H}_2\text{O} \rightarrow \text{HCO}_3 + 2\,\text{H}^+ + 2\,e$	E'_0 "	$-$ 0.410

In this series, the electroactive system, reduced on the oxidation of formate, is oxidized on the reduction of pyruvate.

Another interesting example is given by Szent-Györgyi (283). In the

presence of their respective activating enzymes and of the reversible systems phosphoriboflavin-protein, cytochrome *c* and "cytochrome oxidase," malate is oxidized to oxaloacetate, fumarate reduced to succinate, and succinate reoxidized to fumarate. There is here the following oxidation-reduction series (on the assumption that cytochrome *a* equals cytochrome oxidase):

Ferricytochrome $a \rightarrow$ Ferrocytochrome $a + 1\ e$	$E'_0 = +0.29$ volt
Ferricytochrome $c \rightarrow$ Ferrocytochrome $c + 1\ e$	$E'_0 = +0.255$ volt
$Fumarate^{=} + 2\ H^{+} + 2\ e \rightarrow Succinate^{=}$	$E'_0 = 0$
Phosphoriboflavin protein $+ 2\ H^{+} + 2\ e \rightarrow$ Red flavoprotein	$E'_0 = -0.063$ volt
$Malate^{=} \rightarrow Oxaloacetate^{=} + 2\ H^{+} + 2\ e$	$E'_0 = -0.169$ volt

Interspersed in this series are two electroactive systems: (1) phosphoriboflavin-protein and (2) cytochrome *a* (the "cytochrome oxidase"), the energy of oxidation being transferred stepwise.

The Mechanism of Enzymatic Oxidations. Foodstuff under the conditions existing in living cells is not oxidized by mild oxidizing agents. On collision with a specific protein molecule, the activating protein (dehydrogenase, apodehydrase, *Zwischenferment*), it becomes *activated*, i.e., a change in its electronic structure occurs, by which the electrons become ready to be transferred through the oxidation-reduction series. In the simplest type of oxidation enzyme the activated substrate may be oxidized directly by molecular oxygen. In such a case the series would be made of only two elements, the release of energy being made wastefully, suddenly. Keilin and Hartree (170) believed that uricase amino acid oxidase, and xanthine oxidase are examples of this group. Although such a type may exist, effort must be made to find the systems which mediate the transfer of electrons between the oxidizable substrate and molecular oxygen. In fact, Warburg and Christian (319) and Ball (18) have recently isolated d-amino acid oxidase and xanthine oxidase, which contain two systems: the activating protein and alloxazin-adenine-dinucleotide. Davidson (75a) has found that uricase contains 0.15 to 0.2 per cent iron. This group may then be considered doomed to disappear.

In the second type, the oxidizing enzyme is made up of two components, the activating protein and the electroactive system: d-amino acid oxidase and xanthine oxidase are examples of this group. The activated substrate is oxidized by alloxazin-adenin-dinucleotide; reduced alloxazin is oxidized by molecular oxygen.

The third type requires the presence of an electromotively active

system which will transfer electrons from the oxidizable substrate through the cytochrome system to oxygen. The length of the series will be determined by the number of hemochromogens existing in the system. Succino-oxidase may be given as an example. In the course of the phylogenetic evolution of the cell an oxidizing enzyme may add more components. Thus α-hydroxyoxidase in yeast and gonococci require only the activating protein and electroactive catalyst (Green and Brostreaux (135), Barron and Hastings (32)), but in animal tissues the enzyme adds a sluggish reversible system, diphosphopyridine nucleotide, as another component (Banga and Szent-György (22) Green and Brostreaux (135)).

A fourth type of enzyme requires the presence of two reversible oxidation-reduction systems: one, of the sluggish type, non-autoxidizable by atmospheric oxygen; the other, of the electroactive type, autoxidizable by atmospheric oxygen. The well-known Warburg system which oxidizes hexosemonophosphate may be given as an example. Activated hexose monophosphate is oxidized by triphospyridine nucleotide; reduced triphosphopyridine is oxidized by phosphoriboflavin-protein; reduced flavoprotein is finally oxidized by oxygen. The existence of this type of oxidation seems limited to cells with no iron-porphyrins.

A fifth type of oxidation requires the presence of four reversible systems: two sluggish, a pyridine nucleotide and cytochrome *c*; two electroactive, alloxazin and a hemochromogen (Warburg's Atmungsferment or Keilin's cytochrome oxidase). Theorell has shown that at the low oxygen pressure of tissues the direct oxidation of phosphoriboflavin-protein by oxygen is not very rapid, but that reduced flavoprotein can reduce cytochrome through which the reaction proceeds more rapidly. To this type may be grouped all those systems studied by Green and Euler where the enzyme components are: protein, pyridine nucleotide, the cytochrome system.

Catalase, peroxidase, and polyphenol oxidase seem to require no activating protein. The prosthetic group, iron-porphyrin or copper form complex compounds with the protein, the complex being reduced by the oxidizable substrate, and the reduced complex being oxidized by molecular oxygen (VI type).

As the living cell becomes more complicated and leaves the path of fermentation for that of oxidation, it develops a series of reversible systems of graded energies, systems which deliver smoothly the energy provided by the final reduction of oxygen. The series of oxidation-reduction systems of the cell becomes longer and longer.

Figure 1 gives the oxidation-reduction series as found in heart muscle,

made up of electroactive systems, sluggish systems, and enzymatic-sluggish systems.

Decarboxylative Oxidations. The end products of cellular respiration are CO_2 and H_2O. Oxygen is taken up and CO_2 is given out. In which

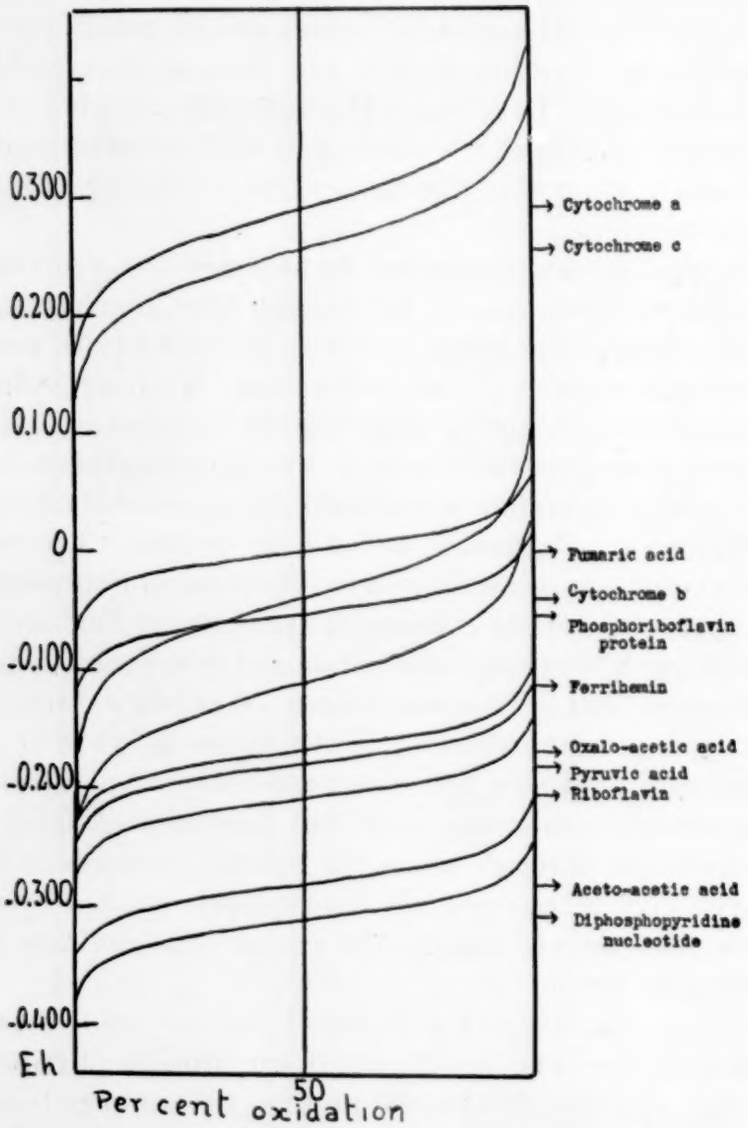


Fig. 1. Reversible oxidation-reduction systems present in mammalian heart. E_h in volts; pH 7.0

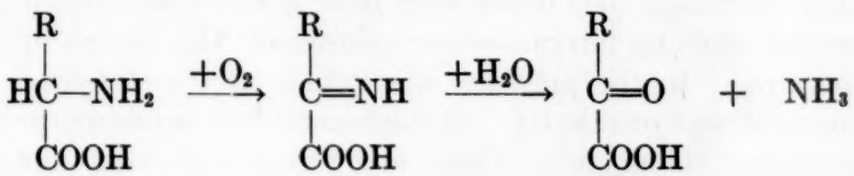
steps of the oxidation-reduction series this CO_2 is given out we barely know. Some of this CO_2 may come through intermolecular oxidation-reductions of ketonic acids (Krebs and Johnson (191), Elliot et al. (91, 92)); some by interaction between α-ketonic acids and amino acids,

as Braunstein and Kritzmann (50) have shown; some by the oxidation of saturated fatty acids; some by the direct oxidation of pyruvic acid, the main intermediate product of carbohydrate metabolism. The decarboxylative oxidation of pyruvic acid was demonstrated with the aid of bacteria (gonococci) which oxidized it to only acetic acid and CO_2 (Barron and Miller (38)). This process required an activating protein and an iron compound which probably acts as a chain-reaction catalyst.

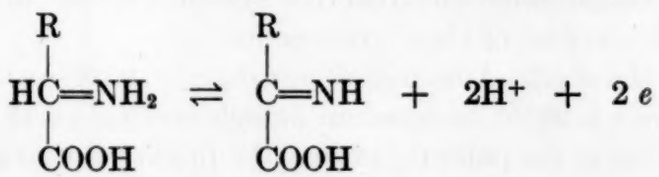
Peters and his co-workers (250) have contributed greatly to our knowledge of the mechanism of this oxidation in their studies of the oxidation of pyruvic acid by avitaminotic pigeon's brain. The oxidation of pyruvic acid was increased on addition of thiamin (vitamin B_1). Lohman and Schuster's discovery that "cocarboxylase," the "coenzyme" for pyruvic acid decarboxylation in yeast, was a diphosphothiamin, was followed by Lipman's (214) demonstration that this diphosphothiamin takes part in the enzyme complex concerned with the oxidation of pyruvic acid. In experiments with bacteria it has been shown (Barron and Lyman (37)) that thiamin must be phosphorylated before becoming part of the pyruvic acid oxidizing system. It takes part also in the dismutation of pyruvic acid. Is diphosphothiamin, like the pyridine nucleotides, reduced by pyruvic acid and reoxidized by an electroactive catalyst? This possibility, pointed out by Clark (64), has not yet been demonstrated.

Work on other decarboxylative oxidations is sorely needed.

The Oxidation and Synthesis of Amino Acids. The oxidation of amino acids starts with the intermediate formation of imino acids and the final formation of keto acids and NH_3:



The first process is a reversible oxidation-reduction system:



The second process, a hydrolysis, proceeds spontaneously. In the oxidation of d-amino acids (by the enzyme purified by Warburg and

Christian), d-alanine, for example, is oxidized by alloxazin dinucleotide and reduced alloxazin is oxidized by atmospheric oxygen:

(1) CH_3CHNH_2COOH + Alloxazin $\rightleftharpoons$
Reduced alloxazin + $CH_3C{=}OCOOH$ + NH_3

(2) Reduced alloxazin + $O_2 \rightleftharpoons$ Alloxazin + H_2O_2

Of these two reversible systems, the system alanine $\rightleftharpoons$ ammonium pyruvate is an enzymatic sluggish system (Wurmser and Filitti-Wurmser (329)), and the alloxazin system is an electroactive system. By virtue of their reversibility there will be either oxidation or synthesis of amino acid, depending on the values of the equilibrium constants.

In the oxidation of 1(+) glutamic acid (Euler *et al.* (100a)) the oxidative enzyme is made up of an activating protein (dehydrogenase), phosphopyridine nucleotides (triphosphopyridine nucleotide in yeast and *B. coli*, di- and triphosphopyridine nucleotides in animal tissues), and probably alloxazin dinucleotide. There are present in this series the three groups of reversible systems: (1) enzymatic sluggish (glutamic acid $\rightleftharpoons$ NH_3Ketoglutarate + $2H^+$ + 2 e); (2) sluggish (diphosphopyridine nucleotide); (3) electroactive (alloxazin). The potential of system (1) at pH 7.0 (30°C.) is about −0.181 volt (calculated from equilibrium data given by Euler *et al.*). The potential of system (2) at the same pH is about −0.314 volt. The potential of system (3) is not known. These values explain Euler's findings, namely, that diphosphopyridine nucleotide reduced by the oxidation of either alcohol or glucose (activated by their respective activating proteins) will readily reduce ammonium ketoglutarate to glutamic acid. It is Euler's opinion that in the liver (where alcohol and glucose oxidases are abundant) the reaction is oriented towards the synthesis of amino acids. Braunstein and Kritzman (50) found that muscle tissue forms and breaks down amino acids by intermolecular transfer of NH_2 groups by means of an enzyme. In the presence of glutamic acid and pyruvic acid, glutamic acid was oxidized to α-ketoglutaric acid, while pyruvic acid was converted to alanine. There are here (aside from diphosphopyridine nucleotide) two enzymatic sluggish systems reacting undoubtedly through some electroactive system present in muscle. At pH 7.0, the E'_0 values of these systems are:

(1) Alanine $\rightleftharpoons$ Ammonium pyruvate, −0.040 volt

(2) Glutamic acid $\rightleftharpoons$ Ammonium ketoglutarate, −0.181 volt.

From the values of the potentials it may be predicted that glutamic acid will be oxidized and pyruvic acid will be converted to alanine, another method of amino acid synthesis.

In the third kind of amino acid synthesis, in which there is formation of an acetyl derivative (Knoop (185a), du Vigneaud and Irish (298a)), essentially the same oxidation-reduction processes are involved: amino acid is synthesized by a series of oxidation-reductions involving pyruvic acid and NH_3. Pyruvic acid, a breakdown product of carbohydrate metabolism, takes part in the synthesis of amino acids and proteins by its reductive amination, a process which is easily performed because of the positive value of the potential of the reversible system alanine $\rightleftharpoons$ ammonium pyruvate.

Orientation of Reactions. The energy required for the maintenance of cell activities comes from foodstuff. The cell either splits it or burns it. We call the first process fermentation, the second oxidation. The cells belonging to the lowest group in the phylogenetic scale are undoubtedly those capable of "vie sans air," as Pasteur (247) called it, in which the energy requirements are met by fermentation processes. Fermentation is much the simpler of the two processes, but at the same time the less economical because the largest part of the energy of the carbohydrate molecule remains in the lactic acid or the alcohol molecule. The greater energy requirements of morphologically and physiologically complex organisms introduced atmospheric oxygen into the metabolism of living matter and thus respiration started. As soon as respiration appeared we had the problem of orientation of reactions. Aerobic cells in the absence of air split carbohydrate to lactic acid or alcohol; in the presence of air, oxygen is consumed, CO_2 produced, and fermentation stopped. The intimate relation of these two processes, a relation whose quantitative interdependence was discovered by Pasteur (now known as the Pasteur reaction) may be considered the integral of these orientations of reactions. Moreover, the only difference between fermentation and respiration is that the former is a series of phosphorylated oxidation-reductions with no oxygen, while the latter is a series of oxidation-reductions some of them decarboxylative, with oxygen as the final oxidation agent. Where does the linkage between fermentation and respiration begin? The numerous inhibition experiments leaving respiration intact and increasing aerobic fermentation, the so-called inhibition of the Pasteur reaction (Krah (186), Warburg (301), Dickens (79)) have brought no light. Bumm, Appel, and Fehrenbach's (53) claim that reduced glutathione inhibits the Pasteur reaction specifically and reversibly has been denied by Baker (12). Lipmann's (213) conclusions from experiments on the inhibition of fermentation by reversible dyes have found another explanation in

experiments of the same kind by Michaelis and Smythe (233). It has been generally agreed that respiration and fermentation are always united and that carbohydrates cannot be directly oxidized without preliminary anaerobic scission. The discovery of enzymes able to oxidize glucose directly (Boysen-Jensen (49), Müller (236), Harrison (148)) and the fact that iodoacetic acid may inhibit glycolysis without influencing respiration (Lundsgaard (218), Shorr, Barker, and Malani (269)) have been serious objections to the validity of this theory. Though the possibility of direct oxidation of glucose in some cases is admitted, it seems that in most cases glucose is first split before undergoing oxidation.

TABLE 5

The metabolism of carbohydrates

Products formed during glycolysis	Hexose $\xrightarrow[\text{glucose oxidase}]{\text{oxidation}}$
	Hexose monophosphate $\xrightarrow[\text{hexose monophosphate oxidase}]{\text{oxidation}}$
	Triose $\xrightarrow[\text{triose oxidase}]{\text{oxidation}}$ further oxidation through $\nearrow$ C_4 dicarboxylic acid catalysis; $\searrow$ citric acid catalysis
	Pyruvic acid $\xrightarrow[\alpha\text{-ketonoxidase}]{\text{oxidation}}$
	Lactic acid $\xrightarrow[\alpha\text{-hydroxyoxidase}]{\text{oxidation}}$

The link between fermentation and respiration (the reader interested in a review of the Pasteur reaction may see two recent articles by Dixon (83) and by Burk (54)) may be produced at the different steps of the series of reactions of carbohydrate metabolism which in fermentation end either in lactic acid or alcohol. One of these steps may be the oxidation of hexose phosphates; another may be the oxidation of trioses through coupled oxidations with the reversible C_4 dicarboxylic acids (Szent-Györgyi (283)) or through the reversible reactions in citric acid oxidation (Krebs and Johnson (192)). The link may occur also after the formation of pyruvic acid, which in the presence of oxygen may be directly oxidized into acetic acid and CO_2 (table 5).

It is at once apparent that the orientation of reactions will depend on

a variety of factors. The number of oxidation-reduction systems making up the series (enzymatic-sluggish systems, pyridine-nucleotides, alloxazins, iron-porphyrin compounds) will determine the degree of complexity of the Pasteur reaction; the orientation will be affected by the oxygen tension (Kempner (179) Leiner (210)), temperature (Kubowitz (193), Nakashima (238), Dixon (82)), concentration of electrolytes (Ashford and Dixon (10), Chang, Gerard, and Shaffer (58)), and hormones (Cohen and Gerard (69)).

To demonstrate the complexities which surround the orientation of reactions in living cells, the metabolism of pyruvic acid may be taken as an example, pyruvic acid being selected because it occupies a central position in oxidation as well as in fermentation. It is the primary oxidation product of triose and the mother substance of lactic acid as well as of alcohol. Activated by the activating proteins of oxidation enzymes, it becomes extremely reactive. If a cell performs its respiration through a small oxidation-reduction series, for example, *Streptococcus hemolyticus*, pyruvic acid is oxidized to acetic acid and CO_2 in the presence of oxygen (Barron and Jacobs (35)), while in its absence it suffers an intramolecular oxidation-reduction splitting into acetic acid and formic acid. In another cell, gonococcus, where there is an enzyme for the reversible oxidation-reduction system ($Pyruvate^- + 2\,e + 2\,H^+ \rightleftharpoons Lactate^-$), in the presence of oxygen pyruvic acid is oxidized as before into acetic acid and CO_2; in the absence of air it is reduced partly into lactic acid and oxidized partly into acetic acid and CO_2 (Krebs (190)). It is obvious that pyruvic acid in these cells will orient its metabolism according to the oxygen tension, pH, concentration of reactants, and other factors which may influence the direction of these reactions. In more highly differentiated cells (muscle or liver), pyruvic acid may combine with amino acids reversibly (the amino group and 2 H of glutamic acid are transferred to pyruvic acid with formation of alanine (Braunstein and Kritzmann (50)); with other ketonic acids (Krebs and Johnson (190), Weill-Malherbe (319)). As the number of oxidation enzymes in the cell increases, the reactivity of pyruvic acid apparently increases for there will be an orientation of reactions of the first split products of pyruvic acid, giving puzzles for the imagination of those working in intermediary metabolism. (See the interesting papers of Elliott et al. (91, 92).)

If pyruvic acid, a split product of carbohydrate metabolism, can orient its cell reactions in so many different ways, it is easy to understand why the mechanism of the Pasteur reaction is still unknown.

CONCLUSION

In this review, cellular respiration has been considered in the light of thermodynamic and kinetic studies as well as the extensive research on the isolation of the oxidation enzymes. From these studies it seems reasonable that oxidizing enzymes in general are made up of two components, a protein and one or more reversible oxidation-reduction systems, the protein combining with varying degrees of affinity with the reversible system. When a group of these enzymes and their oxidizable substrates act together (oxidation-reductions in glycolysis, coupled oxidations, Szent-Györgyi's C_4 carboxylic acid catalysts, Krebs' citric acid catalysis, etc.) the oxidation reactions proceed, as a rule, in a series, the system of more negative potential being oxidized by the reduction of the system of more positive potential, and so on, in a series of graded steps up to the reduction of molecular oxygen. So, like locks in a canal, these reversible systems release gradually the oxidation energy.

The reversibility of many of these oxidation-reduction reactions (systems found among the oxidation products of carbohydrates, amino acids, and fatty acids) is one of the most important factors determining the orientation of reactions. According to the conditions which regulate the values of the equilibrium constants the reactions may be oriented toward either the oxidation or the synthesis of carbohydrates, proteins, and fats.

There is still, however, considerable work to be done in this field. Our knowledge of the enzymes which produce the oxidation of fats, for example, is nil. Little, too, is known of the mechanisms of those agents, such as hormones and vitamins, which control the speed and the direction of biological oxidations.

When applying these studies with isolated enzymes to living cells, we must guard against unfortunate generalizations, which so often are made as soon as a reversible oxidation-reduction system is discovered. We must proceed with the utmost caution, having always in mind the variables which influence the orientation and speed not only of a given reaction but of all those reactions which in the living cell end in the consumption of oxygen and the production of CO_2.

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BACTERIAL CHEMOTHERAPY

The Pharmacology of Sulfanilamide

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The idea of developing specific chemical substances for the treatment of bacterial infections is by no means a new one; in fact, it is as old as bacteriology itself. In 1881, Koch (97) attempted to cure guinea pigs infected with anthrax by the injection of mercuric salts. Although an amount of mercury was used which was more than sufficient, on the assumption of its equal distribution in the host, to destroy the bacteria *in vitro*, the results were not only entirely negative, but the treatment appeared to shorten the life of the infected animal. These experiments were so discouraging that many believed it would be impossible to find any specific for the infectious diseases. Only in 1904, when Ehrlich's studies (52) in chemotherapy began to yield definite results, did interest in the search for drugs as bacterial chemotherapeutic agents revive. However, despite the advances which have been made since Ehrlich's time in the chemotherapy of protozoan infections, it is probably correct to say that nothing of any practical importance was developed in bacterial chemotherapy until within the last few years when drugs containing the sulfonamide group were introduced.[1] This failure was not due to the lack of experimentation along these lines, but probably to the fact that such investigation is still in the stage of haphazard trial and error. It may be recalled that Morgenroth and Levy's use (139) in 1911 of ethylhydrocupreine to cure pneumococcus septicemia in mice was practically the only instance known of an experimental bacterial infection in animals which could be cured by a drug. Moreover, the

[1] For a discussion of earlier work in this field, the reviews of Dale (40) and Jacobs (91) and the book by Findlay (57) can be consulted. Reference must be made to the more recent work of Feldt (56) and Collier (35) on the use of organic gold compounds in pneumococcus and streptococcus infections in mice, to the work of Browning and his colleagues (15) on anilquinoline compounds, and to the recent work on new quinine derivatives (145, 92, 119).

curative dose was very near the lethal dose, and this remedy when tried in pneumonia in man was found too toxic to be given in sufficient dosage for effective therapy.

The above situation has been radically altered by the discovery of the sulfonamide derivatives as bacterial chemotherapeutic agents. The known scope of effective treatment with these compounds is widening rapidly, and while brilliant success has already been achieved, so much ground remains to be explored that the ultimate consequences of this discovery are beyond prediction. We can only trace briefly the development of this important subject. Gelmo (69) in 1908 in an investigation of a purely scientific nature first described the preparation of the compound, para-aminobenzenesulfonamide (sulfanilamide). A year later, Hörlein, Dressel and Kothe of the I. G. Farbenindustrie prepared the first azo dyes with sulfonamide and substituted sulfonamide groups, and found they were distinguished by greater fastness to washing and milling than those of the corresponding sulfonamide-free products. Eisenberg (53) demonstrated the bactericidal action of certain azo compounds *in vitro* and employed chrysoidine for chemotherapeutic purposes, but not the slightest therapeutic effect could be obtained in infected animals. Heidelberger and Jacobs (85) tried to increase the bactericidal properties of the quinine molecule by linking dihydrocupreine with diazo compounds obtained from aromatic amines, including para-aminobenzenesulfonamide, and in their article they state that many of the azo compounds thus formed exhibit bactericidal properties *in vitro* (like the azo dyes of Eisenberg).

In the laboratories of the I. G. Farbenindustrie at Elberfeld, a series of investigations led to azo compounds with marked bactericidal properties *in vitro* but no action *in vivo* in the mouse. In studies confined to compounds containing the sulfonamide group and tested only by animal experiment, drugs effective against streptococcus infections were discovered (47, 89, 135). In 1932, Mietzsch and Klarer obtained a patent for the azo dye, sulfamidochrysoidine (4-sulfamido-2′:4′-diaminoazobenzene), which was found by Domagk (48) to cure an otherwise fatal streptococcus infection in mice. This compound was put out under the trade name of "Prontosil." A more soluble compound, to be given by intramuscular injection, was described shortly after the appearance of the first product (46). This compound is the sodium salt of 4′-sulfonamidophenyl-2-azo-7-acetyl-amino-1-hydroxynaphthylene-3-,6-disulfonic acid and was called "Prontosil Soluble" and later "Neoprontosil." Levaditi and Vaisman (108), using sulfamido-

chrysoidine, confirmed in the main the findings of Domagk, as also did Nitti and Bovet (141). Tréfouel, Tréfouel, Nitti and Bovet (171) found that a number of azo dyes prepared from para-amino-benzenesulfonamide but of different physical and chemical properties had an antistreptococcus action like "Prontosil"; they found, however, that if other aromatic amines were used in preparing the azo dyes they were inactive therapeutically. On the basis of these facts they proposed the hypothesis that all the active azo dyes, including "Prontosil," were reduced in the organism to sulfanilamide. This led them to the very important discovery that the simple colorless compound, sulfanilamide, was itself highly active as a therapeutic agent in experimental streptococcus infections. Colebrook and Kenny (34) and Buttle, Gray and Stephenson (20) in England confirmed the fact that both the prontosils and sulfanilamide were effective against experimental streptococcus infections in mice, as did Long and Bliss (114) in America.

Even before the appearance of Domagk's paper in 1935, mention of the treatment with Streptozon (Prontosil) of patients with infections appeared in German clinical journals (62, 78, 79, 174, 73) and afterwards numerous favorable results in human infections were reported (164, 2, 163, 161, 65, 99) but it was only with the publication of the paper by Colebrook and Kenny (34) on the marked beneficial effect of the prontosils in puerperal sepsis that much interest was awakened in English-speaking countries in the new drugs.

Since sulfanilamide has been used, both in experimental investigations and in the treatment of infectious diseases in man, much more widely than any other of these newer compounds, since certain other compounds probably act by forming this substance in the host, and since more information is available in regard to its pharmacology, the purpose of the present review with its limited space can be best served by discussing mainly this compound and limiting the discussion of other compounds to such points as bear upon important problems of bacterial chemotherapy. We shall not attempt to deal with the clinical use of this drug or the many important problems that have arisen in this connection, but shall limit our discussion to the experimental results which form the basis for the intelligent use of sulfanilamide and related drugs in the therapy of certain bacterial diseases. In a study of the pharmacology of any chemotherapeutic agent three lines of investigation are important: namely, the effect of the drug on the infecting organism *in vivo* in animals, the effect of the drug on the infecting organism *in vitro* under various conditions, and the effect of the drug

on the host. We propose to discuss the treatment of experimental streptococcus and other infections in animals, the toxicity, absorption, excretion and distribution of sulfanilamide, its action on specific organs and tissues, the relation of chemical constitution to bacterial chemotherapeutic action, and the mechanism of action. The field is so new, however, that it is difficult in many instances to sift conflicting data, to assess poorly conducted experiments on small numbers of animals, or to be at all sure of the answer to many important questions which must furnish the basis for an understanding of the subject. Also, since many of the articles dealing with this problem are of the nature of preliminary reports, conclusions based on these papers may have to be modified in the near future.

Treatment of experimental streptococcus infections. Nearly all of the studies with sulfanilamide (and allied drugs) on experimental streptococcus infections have been on mice. Intraperitoneal injection of 0.2 to 0.5 cc. of a high dilution of a mouse-virulent culture of group A of β-hemolytic streptococci followed (or occasionally preceded) by the subcutaneous or per os administration of the drug with a repetition of the dose at varying intervals has been the usual procedure. Control animals usually die within 48 hours, while treated animals may survive much longer or recover from the infection.

Of the three main classes of streptococci, alpha, beta, and gamma (13), only organisms of the β-hemolytic streptococcus group produce satisfactory experimental infections in mice. Nothing therefore is known about the efficacy of sulfanilamide in alpha- and gamma-streptococcus infections in mice (10). In dogs with injured heart valves, a streptococcus viridans infection is stated to have been cured by sulfanilamide (96). According to Lancefield's classification, the β-hemolytic streptococci comprise nine groups designated by letters of the alphabet. In mice infected with mouse-virulent organisms of group A, composed almost exclusively of strains of human origin, sulfanilamide has been found to exhibit a pronounced therapeutic effect (34, 20, 114). According to Buttle, Gray and Stephenson (20) protection can be obtained with sulfanilamide against serologically different types of group A. Few experimental studies have been made on infections in mice with β-hemolytic streptococci belonging to groups other than A. Some protective value of sulfanilamide in mice infected with groups B and C organisms has been reported (10, 131, 166) but the results were not comparable to those obtained in mice infected with group A strains. These experiments are, however, so limited in scope that definite con-

clusions cannot be drawn. No data are available for infections in mice with other groups.

It was stated early that a necessary condition for successful therapy in mice was the use of a strain of hemolytic streptococci of high virulence. Few details of the experiments on which this conclusion is based have been given and most of the observations have been made with the original drug—"Prontosil." Nitti and Bovet (141) were the first to limit the action of the drug to mouse infections with highly virulent strains: their finding is confirmed by Colebrook and Kenny (34), Long and Bliss (114), Bloch-Michel, Conté and Durel (11), and Rosenthal (156). On the other hand, Levaditi and Vaisman (105), Gley and Girard (72), Bürgers (16), and Mellon, Gross and Cooper (132) found protection in mice against strains of low or moderate virulence. Since it is known that even with a highly mouse-virulent strain of streptococci protection with sulfanilamide may be obtained against 10,000 but not against 1,000,000 L.D. (20), and that *in vitro* a bacteriostatic or bactericidal action can be demonstrated only against a limited number of organisms, it would appear from the above results that the important factor in successful sulfanilamide therapy in mice is not the number of L.D. given, but the number of organisms used for infection. Dawson and Hobby (42) found that whereas protection cannot be obtained with a culture of low virulence when given in undiluted form, it can be obtained against the same strain when mucin is used to reduce the L.D. In addition, it is known that better therapeutic results are obtained with a culture of moderate than with one of high mouse virulence provided only small numbers of bacteria are injected.

An interesting and important observation from the standpoint of the mechanism of action of these drugs is the fact that treated mice may die from streptococcus septicemia a long time after treatment has ceased and without showing any symptoms in the interval. Thus, Levaditi and Vaisman (105) had mice dying 18 to 19 days after apparent cure of streptococcus infection, and Long and Bliss (113) report such delayed deaths even after 63 and 128 days.

Such factors as the strain of streptococcus, the size of infecting dose, the time after infection of commencing treatment, the dosage of sulfanilamide used, the interval between doses, and the length of the period of therapy appear to be of importance for the therapeutic effect obtained. The information available on these points is rather meagre and has been obtained usually on an insufficient number of mice to render the results conclusive. It is certain that repetition of a dose several times is much

more effective than a single dose (20, 55). Results can also be found to show that large doses given repeatedly are more effective than smaller ones (20, 23, 128), but no data are available to show what the optimal therapeutic concentration of sulfanilamide in a mouse's blood should be if maintained constant for several days. It is not yet certain whether a continuously maintained low concentration in the blood is more effective than repeated rises of concentrations to high levels with very little or no drug present in the intervals, but such data as exist would indicate that the best therapeutic effect results from the maintenance of a constant blood concentration for several days. The increase in effectiveness of large doses may be due to the longer maintenance of an effective blood concentration of the drug. Little can be said at present about the interval between doses; as stated above the evidence appears to indicate that a constant concentration of drug must be maintained in the blood and tissues for several days in order to obtain the maximum therapeutic effect. In most experiments on mice, the first dose of sulfanilamide has been given immediately after infection. There is good evidence, however, that the drug is still effective when the first dose is given several hours after infection and at a time when a positive blood culture is obtained (20, 114, 131), but due to the small numbers of mice used in most of these experiments, it is impossible at present to assess quantitatively the effect of delaying treatment.

Very few investigations have been concerned with the efficacy of sulfanilamide in experimental streptococcus infections in animals other than the mouse. Mention may be made of the use of the rat by Montestruc (137, 138), Meunier and Nitti (134) and Adolph and Lockwood (1), of the guinea pig by Mellon, Gross and Cooper (131), Seastone (166), and Habs and Bader (80), and of the rabbit by Domagk (48), Nitti and Bovet (142), Montestruc (137), Bürgers (16), Gay and Clark (68), Schaffer (160), and Kolmer, Raiziss and Rule (98). In general, one can conclude from the above studies that sulfanilamide is an effective therapeutic agent in β-hemolytic streptococcus infections in these animals. It is to be noted that in group C infections in the guinea pig and rabbit excellent therapeutic results are reported. Also, it is of interest to note that acute infections but not chronic ones can be successfully treated in the guinea pig (166, 80).

Treatment of other experimental bacterial infections. Although the prontosils and sulfanilamide were at first supposed to be specific against the β-hemolytic streptococcus, subsequent investigations have shown

that these drugs (or allied compounds) may be effective against infections due to several different genera of bacteria, at least one due to a protozoa, and possibly against certain virus infections. Definite evidence has been presented for the efficacy of sulfanilamide in experimental mouse infections due to meningococcus (20, 149, 158, 14, 109, 175, 55), gonococcus (110, 30, 31), E. typhi and paratyphi B (22), Clostridium welchii (112), Sonne strain of dysentery bacillus (18), and a staphylococcus (49, 18, 133, 55). Temporary protection only, with delay of death, but not recovery, was found for mice infected with S. aertrycke, Friedländer's bacillus, P. pseudotuberculosis and P. septica (22).

In regard to pneumococcus infection with types I, II and III in mice, there is agreement of opinion that the drug possesses some activity; different investigators do not agree, however, as to magnitude of the effect. Buttle, Gray and Stephenson (20), Buttle, Parish, McLeod and Stephenson (22), Bürgers (16), Long and Bliss (115), Whitby (175), Feinstone, Bliss, Ott and Long (55) report that although death may be delayed in treated mice, recovery does not occur. Rosenthal (156), Cooper, Gross and Mellon (37), and Fourneau, Tréfouel, Mme. Tréfouel, Nitti and Bovet (64) found not only prolongation of life but a certain percentage of recovery; the fact that these observers used rather small infective doses and strains of low mouse virulence, and in some cases infected subcutaneously and not intraperitoneally appears to explain the discrepancy. At any rate, sulfanilamide is much less active against the pneumococcus than against the hemolytic streptococcus in mice. An exception to this is the finding by Schmidt (162) of an excellent therapeutic effect in mice with type XIV pneumococcus. In rats, a more resistant animal than the mouse, a more favorable therapeutic effect of sulfanilamide against pneumococcus has been reported by Cooper and Gross (36) and Rosenthal, Bauer and Branham (158). The few studies reported for rabbits (158, 100) indicate that the drug possesses a therapeutic effect against pneumococci.

In regard to other bacterial infections against which sulfanilamide appears to exert a beneficial effect, mention must be made of its inhibitory effect on the development of experimental tuberculosis in the guinea pig (152, 21, 167, 76, 44), of its remarkable curative effect in human urinary tract infections with B. coli, Staphylococcus aureus and albus, and group B β-hemolytic streptococci (90, 95, 86, 113), and of the highly suggestive results obtained in several cases of undulant fever (77, 12). Few reports have appeared of its effect on experimental

protozoan diseases. Buttle, Gray and Stephenson (20) state that no protection is obtained with the drug against trypanosome infection in mice or malarial infection in canaries. Campbell (24) reports negative results in rabbit syphilis. On the other hand, Coggeshall (29) found that sulfanilamide had a prophylactic value and marked therapeutic effect on acute malarial infection in monkeys, but was of no value in experimental bird malaria, and Chopra and Das Gupta (25) also found a sulfanilamide derivative effective in simian malaria. In the virus diseases studied, sulfanilamide as well as "Prontosil" and "Prontosil Soluble" have in the main given completely negative results (106, 94, 118, 102). Rosenthal, Wooley and Bauer (159) report successful results with "Prontosil" but not with sulfanilamide in mice infected with the virus of lymphocytic choriomeningitis, but Ronse (154) and MacCallum and Findlay (116) failed to corroborate their findings. Dochez and Slanetz (45) found sodium sulfanilylsulfanilate effective in the treatment of dogs and ferrets infected with canine distemper, but MacIntyre and Montgomerie (117) could not confirm this result; favorable results were also claimed by Marcus and Necheles (120) for sulfanilamide and "Prontosil Soluble" in spontaneous canine distemper, but neither Plummer, Mitchell and Walker (148), working with the ferret, nor Dickerson and Whitney (43), in naturally occurring distemper in the dog, could corroborate their findings. Climenko, Crossley and Northey (28), using disulfanilamide,[2] and Oakley (144), using the glucose derivative of 4:4′-diaminodiphenylsulfone, found a significant therapeutic action of these drugs on mice infected with a moderate dosage of influenza virus. Levaditi (102, 103), Bär (3), and MacCallum and Findlay (116) found that sulfanilamide and certain other compounds have the power of protecting a certain percentage of mice from active strains of the virus of lymphogranuloma inguinale.[3] Although it is too early to assess the significance of these somewhat contradictory observations, it is quite possible that a beginning in a chemotherapeutic attack such as that on bacterial diseases has also been made on virus diseases. It is probable, however, that only certain drugs of this general group are at all effective in virus diseases.

Although the present review makes no attempt to consider the clinical

[2] This disulfanilamide is not the same compound given this name by Rosenthal, Bauer and Branham (158). For a good discussion of the question of nomenclature of the sulfonamide derivatives see Crossley, Northey and Hultquist (38).

[3] There is some question as to whether or not lymphogranuloma inguinale is a virus disease.

side of bacterial chemotherapy, it is important to realize that the brilliant results obtained with sulfanilamide and allied compounds in experimental infections in animals have been in the main duplicated in the treatment of certain infectious diseases in man. In various types of infections due to the β-hemolytic streptococcus (group A, Lancefield) dramatic cures have occurred from sulfanilamide treatment. The excellent results obtained in hemolytic streptococcus meningitis (with a mortality, under all other forms of treatment, of over 97 per cent) constitute rigid experimental proof of the effectiveness of sulfanilamide in human streptococcus infections. There is also no question of the value of the drug in meningoccus meningitis, gonococcus infections, and in urinary tract infections due to various organisms. In staphylococcus and pneumococcus infections, the evidence of the value of this type of therapy is not conclusive. Sulfanilamide has been tried in all manner of diseases, but it is too early and the cases are too few to assess at present its value in most of these.

Toxicity and pharmacological effects on specific organs and tissues. Sulfanilamide was used in human beings before much was known of its toxicity, its pharmacological or its pathological effects in animals. While it is well known that drugs may produce in human subjects toxic effects which cannot be predicted from animal experiments, the value of such animal experiments in avoiding harmful effects in human beings is too well known to need comment.

A number of determinations of the toxicity of sulfanilamide for mice have been reported by different observers. Omitting from consideration the data which are obviously inaccurate due to the use of too small a number of animals, different observers have found for the LD_{50} in grams per kilogram for oral administration 4.0 (51), 6.0 (82), 3.8 (124), 6.3 (5), 3.3 (55), 4.8 (136) and 4.2 (178). It appears that the LD_{50} can be taken as about 4.0 grams per kilogram. Halpern and Mayer (82) state that the rat is more susceptible than the mouse, but Murayama and Leake (140) found the LD_{50} to be 10.0, Molitor and Robinson, 6.2 (136), and Wien (178) 3.9 grams per kilogram. Marshall and Cutting (121) found that with the same large dose the blood concentration in the rat was much lower than in the mouse. The LD_{50} for rabbits on oral administration is given as 2.0 grams (150) and 3.0 grams per kilogram (136). Dogs appear to be more resistant (124, 82, 136) while guinea pigs seem to be even more susceptible to the drug than rabbits (82). In regard to the lower vertebrates, the chicken

(155, 27, 179) is peculiarly susceptible to sulfanilamide while the frog is very resistant and the fish quite susceptible (179).

Since sulfanilamide is not given in a single dose to treat infectious disease in man, a determination of the acute toxicity needs to be supplemented by a study of the effect of repeated doses and chronic toxicity. Little information is available in regard to this. It has been found that daily doses considerably larger than the therapeutic dose given for many weeks do not affect dogs or change the growth curve of young rats and that no pathological lesions are present when the animals are sacrificed at the end of the experiment (124, 5, 136). However, Rimington (153) found in rats given sulfanilamide in the food a marked increase in urinary and fecal porphyrin and a slight loss in weight, but no other sign of toxicity; and Hageman (81) in mice found sub-lethal doses administered for some days led to an increase in eosinophiles in the bone marrow and a deposition of hemosiderin in the spleen. Hawking (84) described in cats dying from large doses of the drug degenerative changes, i.e., chromatolysis, in the neurones of the anterior column of the spinal cord and in some of the nerve cells of the cortex and midbrain. Similar changes in the nerve cells of the brain and spinal cord of dogs receiving repeated doses of the drug have been reported (39). Contrary to all other observers, Davis, Harris and Schmeisser (41) report pathological changes in the liver, kidney, spleen, and lung from administration of the drugs to rats.

Dogs given large doses of sulfanilamide (1 to 2 grams per kilogram) exhibit salivation, vomiting, diarrhea, hyperpnea, excitement, muscular weakness, ataxia, athetotic movements, rigidity, hypesthesia, and finally coma. The symptoms in early stages of poisoning resemble those seen in dogs given large doses of ethyl alcohol; those in later stages in many respects are similar to those shown by decorticated dogs. The placing reactions, known to be due to the cerebral cortex, are absent, and the dogs appear to be blind (82, 124, 136). The symptoms shown by other mammals are in the main similar to those seen in dogs; chickens show a condition suggesting peripheral neuritis (155, 27), while the frog shows only a marked depression and paralysis which is partly, if not entirely, peripheral in nature (179).

The above symptoms suggest that in acute poisoning with sulfanilamide the central nervous system is mainly affected. When examined for its effect on smooth muscle, the frog's heart, blood pressure and respiration, it appears inert in concentrations higher than those obtained

in its therapeutic use (84, 179). The stimulation of respiration, depression of the heart, fall of blood pressure, depression of smooth muscle and rise of blood sugar, observed by Supniewski and Hano (170) are undoubtedly due to the use of the very acid hydrochloride of sulfanilamide, and are acid and not sulfanilamide effects, since they can be duplicated with an equimolecular amount of hydrochloric acid (179). A slight depression of renal function occurs with large doses of sulfanilamide, but this is only transient and of no importance (124, 136). Acidosis is produced in dogs by large doses (124).

A number of toxic reactions from sulfanilamide have been reported in patients taking the drug. The dizziness, nausea, headache, excitement, and confusion are probably of cerebral origin and similar to the cerebral symptoms seen in animals. The acidosis and cyanosis also appear to be direct toxic effects of the drug. The fever, skin reactions, hemolytic anemia, and agranulocytosis may be true idiosyncrasies to the drug (124). Although many of the minor toxic reactions are extremely unpleasant, they are not dangerous and do not necessarily contraindicate the use of the drug. Certain reactions (e.g., anemia and agranulocytosis) are extremely serious and demand discontinuance of the drug. It is interesting to recall Ehrlich's remark at the 1913 London Congress of Medicine that one must be prepared to run certain risks in order to do effective chemotherapy.

Absorption, excretion and distribution. In dogs, sulfanilamide is readily absorbed; a study of blood concentration-time curves after oral ingestion of capsules or tablets of the drug in moderate dosage (0.05 to 0.20 gram per kgm.) as well as direct determinations of absorption in sacrifice experiments indicate that the drug is nearly completely absorbed in 3 to 4 hours (126). When given in solution by mouth, absorption is much more rapid, apparently due to the fact that absorption is negligible from the stomach but extremely rapid from the intestine (122). In the mouse and rat, absorption of the drug when given by mouth is also very rapid (121, 55). In man absorption appears to be similar to that in the dog (126).

In the dog, sulfanilamide is excreted in unchanged form in the urine (126), while in other mammals which have been examined it is excreted partly in the free form and partly as a conjugated compound (66, 126). A conjugated form is excreted by the fish, chicken, mouse, rat, guinea pig, rabbit, cat, pig, cow, horse and monkey (179). This conjugated compound has been shown to be acetylsulfanilamide (the para-acetylamino derivative of benzenesulfonamide) in the human and the rab-

bit (123). Acetylsulfanilamide is practically inactive against streptococcus infections in mice. The acetylation apparently takes place in the liver (83). Fuller (66) found in the mouse an excretion in the free and conjugated form of only 65 per cent after a single dose of sulfanilamide; he believed that the remaining 35 per cent is destroyed in the body. Recently, it has been found that about 90 per cent of injected sulfanilamide can be found in the urine of the mouse in 72 hours, and 1 or 2 per cent accounted for in the tissues (179). Marshall, Emerson and Cutting (126) found 78 to 95 per cent of a dose of the drug to be excreted in unchanged form in the urine in 48 hours in the dog, and that in human beings ingesting the drug in constant dosage per day sulfanilamide balance was frequently attained. Scudi and Ratish (165), in three humans, found after a single dose recoveries of only 51, 53 and 58 per cent, but Stewart, Rourke and Allen (169) in nine patients being given the drug over several days found an average total recovery of 93.2 per cent. The question of whether all ingested sulfanilamide can be accounted for by excretion of the free and acetylated form in the urine or whether it is excreted partly in some other form or destroyed in the organism is an important one. The evidence given above is somewhat conflicting, but it appears that only a small amount is usually unaccounted for.

The excretion of sulfanilamide by the kidney in dog and man is similar to that of urea, but reabsorption by the tubules occurs to a greater extent. The clearance of the drug is definitely increased by an increased rate of urine flow (127). Excretion of the drug is very rapid in the mouse (121); a mouse requires the ingestion, on the basis of body weight, of over ten times as much sulfanilamide per day as a man to maintain the same blood concentration (179).

Sulfanilamide resembles urea and ethyl alcohol in its equal distribution in the organism; it apparently diffuses readily to all tissues and fluids of the body. In the dog, it has been shown that, with the exception of bone and fat, the various organs contain about the same amount of sulfanilamide as blood (125). It is probably present in equal concentration in all parts of the body if concentrations are expressed per unit of water, and can be used like urea to measure the water content of the organism (147). It is present in saliva, pancreatic juice, bile, exudates and transudates in a concentration slightly lower than that in blood, and readily passes into the cerebrospinal fluid (126, 125). As might be expected, it passes from the maternal to the fetal circulation in pregnant animals and humans and is present in the same con-

centration in the fetal and maternal bloods (101, 4, 168). It is curious, in view of the above findings, to note that the blood plasma contains less sulfanilamide than the corpuscles; one would expect just the reverse on the basis of water content. This rapid diffusion and penetration of the drug to all parts of the organism is undoubtedly one of the factors which make the substance a successful chemotherapeutic agent. It is important in dealing with sulfanilamide substitutes to test their diffusion and penetration in the organism.

Relation of chemical constitution to chemotherapeutic action. Two lines of research which are being pursued actively at present have for their objectives 1, to obtain more effective and less toxic drugs to use in place of sulfanilamide, and 2, to obtain drugs which are effective in bacterial infections in which sulfanilamide fails. Since many hundreds of compounds have already been tested, we can study to a limited extent the important problem of the relation of chemical constitution to effective chemotherapeutic action. In general only qualitative conclusions can be drawn, because few comparisons of the effectiveness of different compounds have been made in a really quantitative manner; the number of mice used is frequently too small and, due to differences in absorption and excretion, the doses used do not express the correct relation between the compounds. A comparison of compounds on the basis of the blood concentration necessary for a given therapeutic effect would be much more valuable. Another difficulty is the lack of a standard method for assaying the therapeutic value of new compounds. The strain of organism, the virulence and number of organisms used for infection, the amount and spacing of the dosage, the time after infection of administering the first dose, the length of the period of therapy, and the length of time the mice are observed unquestionably affect the quantitative comparison of two active compounds having different ratios of absorption and excretion. The two general methods in use for assaying a new compound are to compare the compound with sulfanilamide on the basis of average survival time of the treated mice or to compare the mortality rates of groups of mice treated with the compound and with sulfanilamide; these two methods probably do not give the same result with all compounds.

So far, all of these newer compounds which have been shown to be active against bacterial infections contain sulfur.[4] It has been demon-

[4] Buttle and collaborators (19) report slight activity with 4,4′-dinitrodiphenylmethane, 4,4′-dinitrodiphenyloxide and 2,2′dinitro-4-4′-diaminodiphenylmethane. It is also to be noted that the quinine derivatives used by Johnston

strated, however, that the sulfonamide group is not necessary for activity. Many compounds which do not contain this group and cannot yield it in the body are highly effective as bacterial chemotherapeutic agents. Thus mercaptans (71), disulfides (64), sulfinic acids (70), sulfonic acids (20), monosulfides, sulfoxides, and sulfones (63, 64, 23) containing an aromatic nucleus have all been reported to have definite therapeutic activity against streptococcus infection in mice. With few exceptions, all of these compounds which are active contain a nitro, amino, or substituted amino group in the para-position to the sulfur.[5]

Considerable information is available concerning the effect of changes in the sulfanilamide molecule on antistreptococcus activity (20, 172, 75, 135, 7, 38). Little or no activity is found in mononuclear compounds in which either the amino group is replaced by some other group or the sulfonamide group by groups not yielding a sulfonic acid on oxidation. An apparent exception to this is the activity of compounds where the amino group is replaced by a nitro group or azo linkage. This is to be explained by the ready change of such compounds to an amino compound in the organism. A shift of the amino group to the ortho or meta position results in loss of activity; also, a third group in the benzene ring results in loss or lowering of activity. Substitution of the amino group by alkyl, aralkyl, substituted alkyl or aryl, acyl, and alkylidene groups in general results in lowering or loss of activity. However, substitution on the amide nitrogen has a variable effect. Methyl and ethyl groups have little effect; higher alkyl groups decrease the activity, and p-amino- or p-nitrophenyl groups are stated to increase it. Compounds of the type of sulfanilylsulfanilamide (or polycylic chains of the same general type) have been found active; while with substituents in the parent ring the highest activity is found in the para-derivative, carboxy or sulfonic acid groups in the ortho position in the second ring give the greatest activity. Derivatives of the true disulfanilamide type have been stated recently to be active.

The question of whether or not a real specificity for different organisms exists cannot be answered dogmatically at present. The difference in activity of various compounds against organisms like streptococci, pneumococci and gonococci may be a quantitative rather than a qualitative

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et al. (92), the anilquinoline compounds of Browning *et al.* (15), and the gold compounds of Feldt (56) and Collier (35) contain no sulfur. It is unlikely that sulfur is a necessary element in a bacterial chemotherapeutic agent.

[5] However, diacetyldihydroxdiphenylsulfone is also stated to be active (104).

difference. The fact that diaminodiphenylsulfone (23) and 2-sulfanilylaminopyridine (176) are both effective against streptococcus infections, but infinitely more effective against pneumococcus than sulfanilamide would argue for some sort of specificity, as would the limited data available on the treatment of experimental virus infections.

Mechanism of action. The intelligent use of any drug demands that its mechanism of action shall, if possible, be understood, and the logical method of attacking the problem of sulfanilamide substitutes for use in place of sulfanilamide or in infections in which this drug fails would appear to be the discovery of how sulfanilamide acts. When one realizes that in the chemotherapy of no protozoan disease is there as yet any satisfactory and generally accepted explanation of the mode of action of the drug, it is not surprising that in the short time that intensive work on the sulfonamide derivatives has been pursued no satisfactory explanation of the mechanism of action has been found. However, there is reason to believe that in many ways the attack upon the mechanism of action may be more fruitful of results in the case of bacterial than in that of protozoan chemotherapy.

Obviously, in a consideration of this problem, three factors are involved, the drug, the infecting organism, and the host. The first step in any explanation of the chemotherapeutic action is to decide to what extent the reaction between drug and bacteria is sufficient and to what extent, if any, the tissues of the host play a rôle either in changing the drug or in removing the organisms by the so-called defense mechanism. A study of the antibacterial action of the drug *in vitro* under different conditions is a necessary preliminary to studies in the infected animal.

For the original "Prontosil" and "Prontosil Soluble," all observers agree that no effect is exerted even by high concentrations of these drugs upon the streptococcus *in vitro* (48, 106, 34, 114). Indeed, Domagk's original discovery of the effect of "Prontosil" was due to the idea that compounds which were not bactericidal *in vitro* might be so *in vivo* and vice versa. With sulfanilamide, however, the situation is quite different, as most observers are convinced that bacteriostatic or bactericidal effects can be demonstrated *in vitro* with concentrations of the drug of the order of those occurring in the blood and tissues of patients undergoing treatment. Different workers have reported, as the result of their *in vitro* experiments on streptococci with concentrations of sulfanilamide which can be attained in the blood of patients,

no effect (157, 16, 59, 49), bacteriostatic (33, 114, 68, 129, 146, 50) or bactericidal effects (33, 143, 129, 58, 88, 146, 177).

The marked discrepancies shown by these *in vitro* studies are due in the main to differences in the conditions of the experiments. The strain of streptococcus, the size of the initial inoculum, the composition of the media, and the temperature are now known to have a definite effect on the result obtained in *in vitro* tests with sulfanilamide. Bliss and Long (8) state that strains of streptococci belonging to group A (Lancefield) are more susceptible to the *in vitro* action of sulfanilamide than those belonging to other groups, group D strains being highly resistant; Hoare (88) found two resistant types in the twenty-one types—all of group A which were tested. Colebrook, Buttle and O'Meara (33) state that in broth at 37° a concentration of 10 mgm. per cent sulfanilamide inhibited the growth of a small inoculum, but that 1000 mgm. per cent did not affect the growth of an inoculum of 300,000,000 organisms, but did have an effect on one-tenth this number. This dependence of the effect of the drug upon the initial concentration of bacteria has been noted by many workers, and probably explains the lack of any bacteriostatic or bactericidal effect noted by certain investigators. The composition of the media used to make the test can be seen to be a very important factor from the following observations. Bactericidal effects have been reported by Nitti, Bovet and Depierre (143) with very small inocula in Martin peptone broth with 10 mgm. per cent; by Mayer (129) in salt solution with 20 mgm. per cent; by Osgood (146) in bone marrow culture media with 10 mgm. per cent; by Colebrook, Buttle and O'Meara (33) in human and monkey blood and serum with 6 to 10 mgm. per cent but not in rabbit, guinea-pig or mouse blood. These results were thought to be mainly independent of the leucocytes, and were confirmed by Finklestone-Sayliss, Paine and Patrick (58) and Hoare (88), who state that leucocytes are not necessary for the effect. Bliss and Long (9) could not demonstrate any bactericidal effect of sulfanilamide in a concentration of 100 mgm. per cent in broth or serum broth when kept 48 hours at a low temperature (±4°C.), in the same concentration with large numbers of bacteria suspended in Tyrode's solution in 20 hours at 37°C., or in serum from patients receiving sulfanilamide (containing 5 to 10 mgm. per cent of the drug). Bürgers (17) also states, without giving any details, that the bactericidal action of horse, rabbit, guinea-pig or human serum is not increased by the addition of sulfanilamide. Colebrook (32) has

stated that to obtain the bactericidal effect in blood limited aeration is necessary; under conditions permitting free aeration, a different result is obtained. Buttle (18) and Fleming (60, 61) in their latest reports state that the leucocytes are necessary for any marked bactericidal as opposed to bacteriostatic effect in blood. The addition of a drop of blood to a tube of broth or of a drop of broth to a tube of blood decreases the antibacterial effect of sulfanilamide. Mellon and Bambas (130) found that if a culture is diluted with sodium chloride instead of broth before inoculation, a concentration of sulfanilamide may now be bactericidal whereas with broth dilutions it had no effect.

The recent work of Lockwood (111) may explain some of the discrepancies encountered in working with whole blood and serum. This investigator found that whole blood containing 10 mgm. per cent of sulfanilamide and 0.25 per cent of peptone is bactericidal towards streptococci, but that deleucocytized blood and serum under the same conditions are only bacteriostatic and not bactericidal. However, when peptone is excluded sulfanilamide in serum is bactericidal. This author suggests that sulfanilamide prevents the specialized metabolic activity required of invasive organisms by preventing the utilization of a native protein substrate.

All of the above experiments were presumably carried out at ordinary incubator temperature (37°) and probably no careful regulation was attempted. White and Parker (177) have made important observations concerning the effect of temperature upon the action of sulfanilamide *in vitro*. At 40°, a marked bactericidal effect can be obtained in broth, with concentrations of 10 and 20 mgm. per cent of sulfanilamide, while only a bacteriostatic effect is evident under the same conditions at 37°. In view of this finding, it is reasonable to suppose that some of the discrepancies noted in *in vitro* work may be due to variation in the temperature in which individual observers have worked.

An important fact about the action of sulfanilamide *in vitro* is the latent period of 2 to 4 hours before any effect is noticed even if marked bacteriostasis or sterility results in 24 hours. This is shown in experiments carried out in whole blood, deleucocytized blood and in various broth media.

A survey of the literature reviewed above on the effect of sulfanilamide on the streptococcus *in vitro* impresses one with the fact that concentrations of the order attained in the blood and tissues during treatment are definitely bacteriostatic and bactericidal under certain conditions. It is difficult to believe that some such effect does not operate

in the infected animal or man during treatment. To conclude, however, that the whole action is a "parisitotropic" effect on the bacteria in accordance with the simple conception of Ehrlich would be completely unjustified at present. No one familiar with the many exceptions and discrepancies in Ehrlich's theory, particularly as worked out for protozoan chemotherapy (40) could, with the facts available at present, be content with such a simple hypothesis of the mode of action. However, to the reviewer the conclusion appears justified, in the light of present knowledge, that the first effect in the cure of an infected animal or man is a direct action of the drug on the parasite rather than a stimulation of defense mechanisms of the host. Such defense mechanisms may, however, operate as the final stage in the chemotherapeutic process.

Before reviewing the evidence for participation of the host, it is necessary to mention certain results that have been obtained with sulfanilamide in infections of the urinary tract with bacteria other than streptococci. Here, all observers agree that sulfanilamide in such concentrations as occur in urine after its administration has a definite bacteriostatic or bactericidal action on B. coli and certain other bacteria (87, 95, 113). It is probable that in these cases the action of sulfanilamide is a direct antiseptic one on the organism, not necessitating the participation of the host, but it is only fair to state that Kenny *et al.* (95) claim some strains of B. coli to be resistant *in vitro* and not *in vivo*. With the marked effect of slight changes in media and temperature in *in vitro* studies, further experiments are necessary along this line before final conclusions can be drawn as to the mechanism of action of sulfanilamide as a urinary antiseptic.

Domagk (48) in his first paper showed that "Prontosil" therapy brought about phagocytosis of the hemolytic streptococci in infected mice; Levaditi and Vaisman (105, 107, 108) confirmed this and stated it was due to injury or destruction of the capsules of the organism by the drug. Neither Colebrook and Kenny (34) nor Long and Bliss (114) could confirm this observation as regards the capsular changes. In mice infected intraperitoneally or in rabbits intrapleurally with hemolytic streptococci, there is a decrease in the number of organisms and increase in phagocytosis in the treated animals compared to the controls, but no change in the virulence of the drug-treated organisms (9, 68, 67, 132), although morphological changes, which persist only as long as the organisms are in contact with sulfanilamide, have been described (68, 111). These observations are taken as indicating an *in vivo* bac-

teriostasis by the drug sufficient to allow the phagocytes to eradicate the infection.

At present, it seems to the reviewer that the balance of evidence indicates that sulfanilamide affects the invading organism in the sense of producing a bacteriostatic or bactericidal action which in many cases is not sufficient to effect sterilization without the coöperation of the defensive reaction of the host. It is probable that under different conditions of infection and with different concentrations of drug present, the relative rôle played by these two factors varies. Be that as it may, we have as yet very little idea of how sulfanilamide acts upon bacteria; such phenomena observed *in vitro* as the latent period of several hours, the effectiveness of low concentrations of sulfanilamide on a small inoculum, but not on a large inoculum, and the marked influence of changes in medium and temperature still await explanation. Grace (74) records the extremely interesting observation that sulfanilamide in low concentrations acts as a plant hormone. Barron and Jacobs (6) and Chu and Hastings (26) found no effect of the drug on the oxygen consumption of isolated tissue or bacteria, but their observations extended only over a period of an hour, and resting bacteria were used. It seems clear that sulfanilamide does not act like the ordinary antiseptics in destroying resting bacteria, but acts only on actively growing organisms and interferes in some way with their metabolic activities.

Mayer (129) in an attempt to explain the latent period and certain other facts in regard to the action of sulfanilamide investigated the oxidation products of this substance. He found p-hydroxylaminobenzenesulfonamide to be about 100 times as active *in vitro* as sulfanilamide and to exhibit this action without a latent period. On the basis of these observations, he assumes that this substance, formed from sulfanilamide *in vivo*, is the active agent. In work in this laboratory, which had been begun along the same line with the hydroxylamine derivative of benzenesulfonamide, we have been unable to obtain any definite evidence that the hydroxylamine derivative is the active agent, although our observations do not rule out this possibility. We have confirmed the fact that this substance is much more active than sulfanilamide *in vitro* and acts without a latent period.

Although it is not our intention in the present review to discuss the many sulfanilamide substitutes which have been described, it is obviously necessary to discuss briefly the two original compounds, "Prontosil" and "Prontosil Soluble," the introduction of which initiated the renaissance in bacterial chemotherapy. As stated before, all observers

are agreed that these compounds have no effect upon the streptococcus *in vitro*. The Tréfouels, Nitti and Bovet (173) proposed the hypothesis that the "Prontosils" had no action as such, but acted only after reduction in the body to sulfanilamide. Colebrook and Kenny (34) found that the blood and serum of patients treated with the "Prontosils" showed bacteriostatic effects against streptococci. It was also shown that strong reducing agents would reduce "Prontosil" to a substance possessing bacteriostatic properties *in vitro* (33, 114). Kellner (93) found that when "Prontosil" was given by mouth an increased color developed in the urine after diazotization and coupling with β-naphthol, which was taken to indicate the presence of sulfanilamide. However, since "Prontosil" itself gives an increased color under these conditions, the evidence is not satisfactory. Fuller (66) showed conclusively (by isolation from the urine) that a considerable amount of sulfanilamide is excreted in the urine[6] when "Prontosil" is given by mouth or "Prontosil Soluble" by injection, but did not feel that sufficient data were available to decide if the "Prontosils" acted entirely through the sulfanilamide formed. Gley and Girard (72) found a carboxy derivative of "Prontosil" to be just as active as sulfanilamide although it could yield only a third as much sulfanilamide; they used this as an argument against the theory of "Prontosil" acting through the sulfanilamide formed. Others (128, 151, 20) have found the prontosils as active as sulfanilamide, weight for weight. Feinstone, Bliss, Ott and Long (55), in a large series of mice, showed that molecule for molecule, "Prontosil Soluble" and sulfanilamide were equal in therapeutic efficiency, if given by injection immediately after infection and twice thereafter on successive days. They also found the blood levels of sulfanilamide four hours after a single dose of molecular equivalents of these two drugs to be the same, although the concentration from sulfanilamide was very much higher before this. This led them to conclude that in the mouse the action of "Prontosil Soluble" can be attributed to its breakdown to sulfanilamide.

The difficulty in interpreting all of these observations is due to the fact that where the drug is given only once a day, the blood sulfanilamide level rises sharply to a high peak and declines rapidly when sulfanilamide is given, but reaches a low level and maintains this in the blood for a longer time when "Prontosil Soluble" is given. As stated before, we do not know the relative effectiveness of these two types of blood sulf-

[6] Engel (54) has shown that liver, kidney, and blood reduce "Prontosil" to sulfanilamide *in vitro*.

anilamide levels and until this is known definite decision about the mechanism of action of the "Prontosils" cannot be made. The data available so far do not eliminate the possibility of the "Prontosils" acting entirely by slow decomposition into sulfanilamide.

In conclusion, there is no doubt that the advent of sulfanilamide and related drugs is of great significance, not only because it has altered, in a favorable sense, the prognosis of hemolytic streptococcus and other bacterial infections, but also because it has established beyond any doubt the possibility of effective chemotherapy for bacterial diseases. Moreover, from recent preliminary reports, it is quite reasonable to expect that chemotherapy may also be highly effective against certain virus diseases.

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THE ULTRASTRUCTURE OF PROTOPLASMIC CONSTITUENTS

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The term ultrastructure as used in this review denotes the submicroscopic organization of cellular and tissue components and includes not only the shapes and orientations of the larger aggregates and micelles,[1] but also the configurations of the molecules which compose the micelles and the various phases of protoplasmic systems. While, strictly speaking, the determination of tissue ultrastructure is a branch of morphology, it is intimately connected with chemistry since it is essentially the morphology of molecular complexes; to learn the details of the ultrastructural organization of such systems it is necessary to know the chemical composition and the physical and chemical properties of the molecules which compose the system. It is a subject of great interest also to physiologists, for just as a knowledge of gross and microscopic anatomy was essential to the development of physiology in the past, so in the modern era with its great advances in the structural chemistry of complex biological substances, a knowledge of cellular and tissue ultrastructure must be obtained if physiologists are to make full use of the recent advances in chemistry in order to discover the mechanisms which underlie tissue and cell function. Obviously before the dynamic aspects of a system, biological or otherwise, can be studied efficiently the structure of the system must be known.

Structure determination should be regarded, therefore, as a means to an end rather than as a final goal in itself. For example, if application of optical methods succeeds in demonstrating the specific configuration of the fibrous protein molecules in resting muscle, it still remains to be determined whether this configuration is altered during

[1] The word micelle has been used rather loosely in recent years to denote structures fundamentally different from those which Nägeli had in mind when he coined the word in 1858. It may, in fact, be wise to discontinue its usage unless a satisfactory definition can be agreed upon for general adoption by chemists as well as biologists. For an illuminating account of this problem and of the micellar theory, see Frey-Wyssling (76, pp. 80–88).

contraction or tension development, and whether a general theory of contractility can be based on such facts. In view of the highly technical nature of the optical methods and the relative expense of the equipment it is desirable that the static and dynamic aspects be developed together rather than as separate investigations by morphologists and physiologists, respectively.

Until the electron microscope is developed, protoplasmic ultrastructure must be investigated by indirect methods since the particles are considerably smaller than the wave length of visible light. Data from polarization, diffraction, and interference methods must be pieced together to obtain an impression of the configuration of the entire system. And in order to interpret the optical data correctly a large body of supplementary evidence must be considered. One must be thoroughly familiar with what is known about the histology and cytology of the system being studied. For example, if one wishes to investigate a given protein structure in a tissue by x-ray diffraction one must know the relative amount and orientation of the other protein components as well. Thus if it is desired to investigate the neurofibrillar apparatus of the nerve axis cylinder by x-rays a nerve must be chosen which has a minimum of connective tissue since the latter might give rise to diffractions similar to those which might be expected from neurofibrils. It is also important that the main chemical entities composing the structure to be investigated be identified and if possible characterized. This may be difficult particularly if the structure is built of complex conjugated protein molecules. It frequently happens that data of the sort mentioned are not available in the literature and, if they appear to be of strategic importance in the interpretation of the optical data, it may become necessary to make independent investigations of these matters. Such supplementary investigations often consume more time than the original optical experiments.

Information of great value may be obtained by a consideration of the optical, physical, and chemical properties of partial systems or artificial models of biological systems. Thus valuable information has been gained about muscle ultrastructure by a consideration of the properties of artificially prepared myosin films and threads; about chromosome structure by observations on nucleic acid and nucleoprotein threads; about the myelin sheath from experiments on lipide myelin forms. Work in recent years on the structure and properties of monolayers and interfacial and built-up films has been most valuable in suggesting structures and processes which may be of importance in

biological systems. But, however suggestive such experiments on models and analogies may be, they cannot demonstrate the structure or process which is unique for the tissue or cell; this can be discovered only by investigation of the biological systems themselves by methods discussed below.

It may also be mentioned that polarized light and x-ray diffraction methods avoid certain objections frequently raised to morphological investigations since the cells and tissues may be examined in many instances in the fresh, unfixed condition. Since it can be shown that in many cases the illumination with polarized light or the brief radiation with x-rays produces no destructive effect on the system, these methods avoid the indeterminacy of the fixation methods of classical morphology, and can be relied on, when properly applied and interpreted, to reveal the structure as it exists in the living cell or tissue.

The optical techniques of use in ultrastructure investigation and their application to biological problems have until recently been most highly developed in Germany. Indeed, neither handbooks describing the use of the polarization optical analysis in biology nor detailed accounts of the results of such investigations are available in English. No attempt will be made in the present review to provide a highly condensed, hence probably unintelligible, summary of the recent German reviews and handbooks. Sufficient technical information will be given to permit the uninitiated reader to gain an idea of the scope and possibilities of the several methods and of the results which have been obtained. Present-day theories of protoplasmic ultrastructure will be reviewed in detail in the case of a few systems, chosen because they are representative and of considerable physiological interest.

I. METHODOLOGY OF ULTRASTRUCTURE DETERMINATION AND INTERPRETATION OF THE DATA. A. *Direct Methods.* Ultraviolet microscopy can hardly be considered a method for the determination of ultrastructure, since the limit of resolution is of the order of 0.06–0.1μ (600–1000 AU). The method has proven useful in biology, particularly because of the fact that certain protoplasmic constituents absorb strongly in this spectral range, hence are visible without the necessity of staining (213). By a microphotometric method Caspersson (38) has made this property the basis of an extremely sensitive analytical tool by means of which the presence and location in cells of ultraviolet-absorbing substances can be determined. Since nucleic acid is such a substance the method has proven very fruitful in determining the structure and composition of chromosomes.

The most promising method of revealing the structure of submicroscopic objects directly is that of the electron microscope. Because of their charge, a beam of electrons may be deflected by a magnetic or electrostatic field. Hence in the electron microscope, electromagnetic coils or charged condensor plates are used in place of the objective and ocular lenses of the ordinary microscope. The electron rays are thus bent and focused in a manner analogous to that in an ordinary microscope and the image is produced on a photographic plate or fluorescent screen. Since the wave length of an electron beam is very small (for a 40,000 volt beam, $\lambda = 0.06$ AU), the resolution theoretically attainable is many thousands of times greater than the maximum of the ordinary microscope. Useful magnifications five to ten times that of the ordinary microscope have already been obtained and very much higher magnifications will doubtless be possible as the technique is improved.

Unfortunately the use of the method in biology is severely limited by the fact that the object to be examined must be dried, placed in a high vacuum, and subjected to an electron bombardment which is sufficient to destroy most biological objects. There is also a serious difficulty in focusing the beam on any given small region of a tissue if really high magnifications are to be sought. However, some of these difficulties will doubtless be overcome as the method is developed. Indeed, results of considerable biological interest have already been obtained (see, for example, 58, 28, 29, 91). It should be pointed out that if this direct method can be made as effective as seems theoretically possible, the indirect methods which involve polarization, interference and diffraction may be completely superseded for the analysis of objects which can be dried without too much artifact production.

A modification of an electron microscope (108) recently introduced by Scott promises to furnish useful information of an analytical sort. Instead of bombarding the tissue with an electron beam the tissue is made to emit electrons by the application of heat. Since the emission temperature is fairly critical for individual atoms such as K, Na, Ca, etc., it is possible in this way to determine the location of these substances in cells. Very clear pictures have already been obtained for the distribution of calcium in muscle and other tissues (175).

B. *Indirect Methods.* 1. *Polarization optics.* For an account of the theory and methodology of the polarization optics the reader is referred to books and articles of Schmidt (142, 151, 162), Ambronn and

Frey (6), Köhler (96), and Rinne and Berek (137). A brief definition of the terms commonly used in such work is given in recent reviews by Schmitt (168) and by Schmitt and Bear (171). It is the purpose of the ensuing section to indicate briefly the type of information which can be obtained by the method and to facilitate an understanding of subsequent sections on the use of the method for the analysis of ultrastructure.

a. Double refraction (birefringence). A tissue or cellular component is said to show double refraction if, by virtue of an anisotropic arrangement of some of its constituent molecules or submicroscopic particles, it transmits a beam of plane polarized light with different velocities depending on whether the plane of vibration of the light corresponds to certain symmetry axes of the system (such as the long and short axes of a fiber). The system has two descriptive refractive indices rather than one, hence is called birefringent. Numerically the birefringence is equal to the difference between the two descriptive refractive indices, though in practice it is determined by means of a compensator which measures the phase difference, or retardation, of the one ray behind the other in passing through the object. The compensator is adjusted so that its retardation just matches (in magnitude) and opposes (in sign) that of the object. The birefringence is obtained by dividing this retardation (usually expressed in $m\mu$) by the thickness of the object expressed in similar units. If the refractive index for rays vibrating in a plane parallel to one direction of the object, let us say the long axis of a fiber, is greater or less than the refractive index for rays vibrating in the perpendicular direction, the object is said to show, respectively, positive or negative birefringence with reference to this direction. If the direction of the optic axis (direction along which the object shows no birefringence) is known, the sign should be that referred to this direction. In practice the sign of birefringence is determined by the characteristics of the compensator, which is calibrated with regard to sign as well as magnitude of birefringence.

Birefringence in biological objects is due to one or more of three causes. These may briefly be characterized as follows:

1. Crystalline (Eigen) birefringence is characteristic of systems in which the molecules are arranged as in a regular anisotropic array. Its magnitude is independent of the refractive index of the material surrounding the oriented molecules. In animal cells and tissues the structural components ("Bausteine") are usually of protein or lipide nature and both of these types characteristically show some degree of crystal-

linity as is evidenced by their birefringence. In general, both protein and lipide components show positive uniaxial crystalline birefringence, the optic axis indicating the orientation of the long axes of the molecules. In protein structures this means that the molecular chains extend in directions parallel with the optic axis which is also the long axis in the case of fibers. Nucleoprotein fibers are a notable exception to this rule, showing negative uniaxial crystalline birefringence instead of positive, but this difference is due to the nucleic acid component rather than to the protein component. Lipide molecules frequently have polar groups at one end of the long paraffin chains and non-polar groups at the other end. This configuration is to a large extent responsible for their tendency to orient in smectic fluid crystalline layers, the long paraffin chains being oriented perpendicular to the planes of the layers and the polar ends of the molecules in each layer being in apposition with those of the neighboring layer (37, 121, 106, 104). In protoplasmic systems lipide molecules are frequently associated with protein components and in this case the lipide molecules usually extend with long axes perpendicular to the long axis or planes of the protein chains. The signs of birefringence of the two components are therefore opposed, and this fact aids in diagnosing the chemical nature of the structure. Thus, if a fibrous structure shows crystalline birefringence which is negative with respect to the fiber axis the presence of oriented lipide may be suspected. This diagnosis may be considered as confirmed if extraction with lipide solvents abolishes the negative birefringence or reverses the sign to positive (characteristic of a protein component). The relative change in birefringence which results from lipide extraction is thus a measure of the lipide concentration in the structure. Nucleoprotein fibers also show birefringence which is negative with respect to the long axis. No confusion need occur, however, since extraction of nucleoprotein fibers not only fails to reverse their sign of birefringence or decrease its magnitude but frequently increases the negativity by desolvation.

2. Photoelastic birefringence. Substances, though intrinsically isotropic, may be made anisotropic by subjecting them to directional strains or compression. Such birefringence is seldom encountered in the relatively fluid protoplasmic systems, although in tissues like muscle it may play a rôle (73) and it has been suspected of being of importance in determining orientations in the developing embryo (60, but see 142). Recently it has proven useful in testing the theory (204) that nerve fibers grow out along lines of molecular orientation, the neuroblasts

being grown on a protein gel which has been subjected to mechanical forces, the orientation being detected by the photoelastic birefringence (129, 130). In such studies one must be careful to distinguish between birefringence due to mechanical orientation of anisotropic particles and that due to anisotropy produced by unidirectional mechanical forces acting on an isotropic body.

3. Form birefringence. The submicroscopic particles which compose protoplasmic systems may show birefringence merely because of certain geometrical properties regardless of whether or not the molecules within the particles are themselves oriented. If the particles are small with respect to the wave length of light, are asymmetric in shape, and are preferentially oriented the system will show "form" birefringence. Such birefringence will vanish, however, if the refractive index of the particles is equal to that of the material in which they are suspended. The greater the difference in refractive indices the greater the form birefringence.

Much information of a quantitative as well as qualitative sort may be obtained as to the shapes and relative volumes of such asymmetric particles by application of the theory of O. Wiener (205). Wiener showed that if the long axes of the asymmetric particles are parallel to the optic axis of the system the birefringence is positive; if the axes are perpendicular to the optic axis the birefringence is negative. This is of great diagnostic value, for if a tissue component shows positive form birefringence it may be concluded that the ultramicroscopic particles are rodlets with long axes parallel to the optic axis; if it shows negative form birefringence the particles are platelets with faces lying in planes perpendicular to the optic axis. The Wiener expressions are as follows:

$$\text{Rodlet form birefringence} \propto n_e^2 - n_0^2 = \frac{\delta_1 \delta_2 (n_1^2 - n_2^2)^2}{(\delta_1 + 1) n_2^2 + \delta_2 n_1^2}$$

$$\text{Platelet form birefringence} \propto n_e^2 - n_0^2 = -\frac{\delta_1 \delta_2 (n_1^2 - n_2^2)^2}{\delta_1 n_1^2 + \delta_2 n_2^2}$$

Where n_e and n_o are the refractive indices of the system for the extraordinary and ordinary rays, respectively, n_1 and n_2 are the refractive indices of the particles and the surrounding medium, respectively, δ_1 and δ_2 are the partial volumes of the particles and the surrounding medium, respectively.

Since form birefringence vanishes when $n_1 = n_2$ but crystalline bire-

fringence is independent of n_2, the relative importance of form and crystalline birefringence is determined by a technique introduced by Ambronn (1–5), namely, by immersing the tissue consecutively in fluids of different refractive indices, measuring the birefringence after each fluid has had time to penetrate. Plotting birefringence as ordinates against n_2 as abscissae one may obtain curves such as those shown in figure 1. If the birefringence is due exclusively to the form factor it will be abolished when $n_1 = n_2$ (curve B). In most biological systems both form and crystalline birefringence are present (curves A and C). In this case the magnitude of the crystalline birefringence is given by the ordinate of the minimum of the curve. In curve A we have posi-

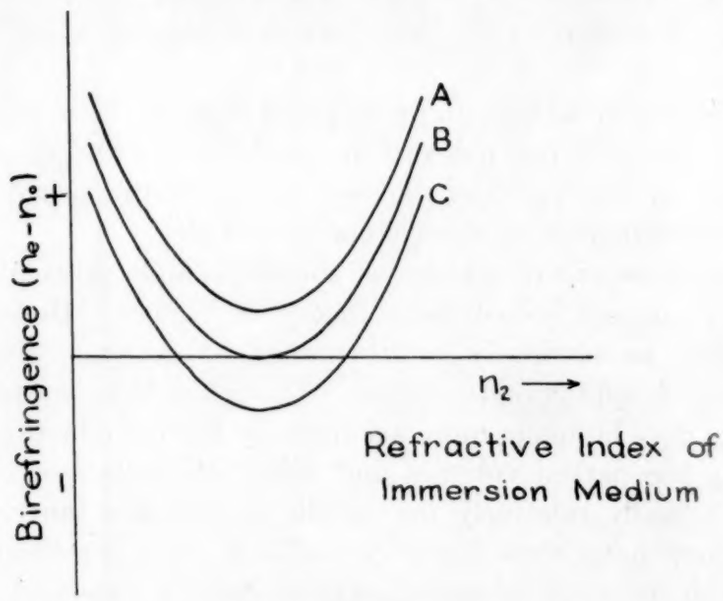

Fig. 1

tive (rodlet) form birefringence and positive crystalline birefringence. In curve C we have positive form birefringence and negative crystalline birefringence. Similarly, we may have negative (platelet) form and negative crystalline birefringence or negative form- and positive crystalline birefringence.

The Wiener relation is also of assistance in determining the relative volume, δ_1, occupied by the oriented particles. In some instances, such as in the case of muscle, sufficient is known about the protein which forms the particles to permit calculation from the form birefringence data and the Wiener relation of the volume occupied by the particles and of the changes in orientation and volume which occur during

physiological activity (see p. 288). In other cases little may be known of the properties or concentration of the substance responsible for the effects. Even in these cases, however, useful information may be obtained. Thus the steepness of the curve of form birefringence depends largely upon the product $\delta_1 \delta_2$ in the Wiener equation. The slope will be maximum when $\delta_1 = \delta_2 = 0.5$. By suitable choice of conditions and of immersion media, δ_2 can be varied within rather wide limits. From the shapes of the curves an estimate can be made of δ_1 under these experimental conditions, and by extrapolation, the value of δ_1 under conditions obtaining normally in the cell or tissue can be determined. A number of biological systems including muscle (188, 118, 202, 203, 125, 73, 74), elastoidin (72), nerve axis cylinder (18), nerve myelin sheath (82), and fibrin (59) have been investigated along somewhat similar lines.

Other useful facts which can be obtained from the form birefringence analysis are the refractive index of the particles (n_1) and the crystalline birefringence of the particles themselves (as distinguished from the crystalline birefringence of the system as a whole).

Biological systems are seldom as simple as that postulated in the ideal two-component mixed body theory of Wiener. However, even when applied to obviously multi-component systems, the analysis leads to useful approximate values. Though seldom actually done, the analysis may be made more accurate by the introduction of terms representing the partial volumes and refractive indices of extra components. Actually relatively few of the possibilities inherent in the Wiener theory have thus far been realized; only the simplest cases involving the extremes of asymmetry (infinitely extended "rodlets" or "platelets" perfectly oriented) have been considered.

b. Double absorption (*dichroism*). As anisotropic structures have different refractive indices for light polarized in certain directions, so they may also possess different absorption coefficients. With plane polarized white light the color of such objects depends upon the relation between the plane of polarization and the distinguishing directions of the object. With plane polarized monochromatic light one may observe extinction in one direction without great effect in the perpendicular direction. The directions for which the maximum effects are observed will usually coincide with the directions of greatest and smallest refractive index of the object. Objects showing this effect are said to be dichroitic or pleochroitic. One Nicol prism only is used, and this merely to determine the plane of vibration of the light.

Again, like birefringence, dichroism may be due to intrinsic factors (crystalline- or Eigen dichroism) or to form factors (form dichroism). In the former the absorbing material is present between the oriented components as crystals or molecules which are themselves oriented. In the latter case the absorbing material is unoriented. By the method of dichroism it is possible to detect anisotropy, even though the oriented particles show no crystalline birefringence and though the refractive index (but not the absorption coefficient) of particles and surrounding medium are the same.

A number of protoplasmic systems show natural dichroism (163), one example being the retinal rods, where the effect is due to the presence of visual purple (153). But the chief biological application comes from making structures artificially dichroitic by impregnating the tissue or cell with substances such as gold, silver, or certain dyes, which are themselves highly dichroitic. Thus it is possible to detect orientation in objects in which the birefringence may be too weak to be detected in other ways; the dichroitic reagent acts as an optical amplifier, as it were.

For more detailed information on the theory and applications of dichroism the reader is referred to the article by Schmidt (146). The methods and equipment are relatively simple and might well be used more generally by the cytologist as well as by the investigator of ultrastructure. Particularly promising is the fact that certain vital dyes confer dichroism to the components of living cells and tissues, hence avoid the production of artifacts unavoidable with other methods.

1. X-ray diffraction. To determine the dimensions, configurations, and orientations of the molecules of which cellular structures are made x-ray diffraction methods may be used, since the wave lengths of x-rays are smaller than the dimensions of the molecules to be studied. Since very good accounts of the theory and methodology of x-ray diffraction are available in English (44, 7, 184, 32, 54, 214), we shall consider in the present section only certain details and precautions which must be observed in the application of the method to biological problems, together with a few points which should be kept in mind in interpreting the significance of the patterns.

In some tissues, such as tendon and nerve, very large spacings have been observed (100–400 AU). To detect such large spacings it is necessary to use long wave length (Kα lines of Cu, Fe, or Cr) x-rays, place the photographic plate at considerable distance (20–40 cm.) from the specimen, use small, well-centered beads to cut out the primary beam

(large beads may prevent the detection of large spacings which would lie close to the central beam). and collimate the beam to a very fine pencil of rays (a divergent beam would mask the presence of large spacings). Instead of using the radiation directly emitted by the tube one may use a Laue beam reflected from a crystal (for copper radiation pentaerythritol gives relatively high intensities (70, 71) though calcite, rock salt, etc., are more stable to x-rays). This has the advantage of furnishing monochromatic and relatively non-divergent radiation, hence very long specimen-to-film distances may be used.

As is well known, x-rays produce injurious effects on tissues and cells when the radiation is very intense or prolonged. It is therefore necessary to demonstrate that the amount of radiation required to produce diffraction patterns from tissues is not sufficient to render the tissue appreciably abnormal. Boehm (23) found that muscle was relatively sensitive to radiation as indicated by alterations in the demarcation potentials. Nerves appear to be less sensitive since radiation sufficient to produce patterns has little effect on action potentials (169).

The distance between the planes producing diffraction effects is calculated from the Bragg relation, $d = \frac{n\lambda}{2 \sin \theta}$. This leads to correct values where the material producing the diffraction has crystal-like regularity of its space lattice but is inapplicable to diffraction in liquids. Since smectic mesomorphic systems have essentially liquid randomness in the planes of the smectic layers the molecular structure within such layers must be determined in other ways (see 134, 201). This is of particular importance in the case of lipide systems. The facts of greatest importance which can be obtained from the diffraction analysis are: 1, interplanar distances; 2, the presence and direction of preferential orientation; 3, the approximate dimensions and degree of asymmetry of the particles or micelles, whose internal lattice structure produces the diffraction (from microphotometric measurement of the widths of the equatorial and meridional diffractions, according to the method of Laue (103); 4, the intraplanar structure, e.g., some information concerning the probable structure of the molecules lying between the confines of the regularly repeated planes (from the relative intensities of the various orders, aided by the methods of Patterson (126) and Harker (83) though these often lead to no unique solution of the structure).

2. Electron diffraction. Analyses of the structure and orientation of organic molecules of biological interest by electron diffraction have been

confined thus far to straight chain molecules which give relatively simple patterns (79, 48). Though more complex substances will doubtless be investigated eventually, interpretation of the patterns of such substances will be extremely difficult. Like the electron microscope, the method suffers from the fact that the material must be thoroughly dried and placed in a vacuum. Because of the efficiency of scattering, however, the exposure time may be very short (patterns obtainable in a few seconds). Moreover, patterns may be obtained from very thin objects, such as a few layers of fatty acid molecules (123, 120, 196, 197, 185, 186). This suggests that valuable information may be obtained by electron diffraction concerning the structure of the mammalian red cell envelope since the latter has a thickness when dried of only 150–160 AU (174). Thicker objects may be investigated by the reflection method. Since, to have appreciable penetrating power, the electrons must have an energy of at least 10,000 volts and since such electrons have very short wave lengths ($\lambda = 0.12$ AU), the method will be of value chiefly in connection with the smaller structure periods. Another difficulty about the technique arises out of its sensitivity. Mere traces of foreign material on the surface of the object may leave a record on the pattern. This has caused difficulty in the analysis of pure compounds and may prove very troublesome with biological objects.

3. Intereference methods with the visible or near-visible spectrum. Tissues such as striated muscle which show a regular and parallel arrangement of striae or planes may act like a diffraction grating for visible light. From the grating equation, $n\lambda = \mathrm{d} \sin \theta_n$, the distance d, between the striae or planes can be calculated, λ being the wave length of the light, n, the order of the spectrum, and θ_η the angle of diffraction of the n^{th} order spectrum. It will be seen that the method cannot be used if d is less than $\frac{1}{2}\lambda$, and is therefore of little significance for ultrastructural investigations. It has been valuable, however, in following rapid changes in d, as in muscle contraction (135, 21, 124, 140, 141).

A number of optical systems which involve interference and diffraction effects have been devised and recommended for the investigation of biological ultrastructure. No attempt will be made to discuss the variety of dark field, ultropak, and combination techniques which have been evolved; much of this work is highly empirical and of little quantitative significance. The Spierer lens (183, 176, 178) was designed to provide a brighter, clearer picture than is possible with the ordinary dark field. Thiessen (195) and Seifriz (177) have observed striae in

cellulose structures, and in cytoplasm, with the Spierer dark field system, which are said to represent aggregates of micelles. It is also said that the existence of stresses and strains in protoplasm is evidenced by the appearance of striae as viewed with the Spierer lens. The structure of muscle has also been investigated by this method (99, 100, 101). Clifford and Cameron (49) state that measurements from Spierer micrographs furnish no information concerning any unit characteristic of the material under observation, for it is inconceivable that the unit would have a dimension common to so many and so diverse substances.

The most recent method depending on interference in the visible spectrum is that of Schmitt and Waugh (174) by which the thickness and chemical composition of the red cell membrane has been determined. With this method the red cells are deposited on a specially prepared slide, hemolyzed in distilled water or other solution and dried. The intensity and color of the light which is reflected when illumination is at normal incidence depends upon the thickness and refractive index of the membrane material. Since the refractive index of the red cell envelope is similar to that of built-up stearate films made according to the method of Blodgett (22) the thickness of the dried double membranes may be determined by matching them for color and intensity against standard stearate step films (the step thicknesses of which are known). The glass slides are of high refractive index and are painted on the under side to prevent reflection of light from this surface. The cells are viewed with the aid of a vertical illuminator system similar to that used in the study of metal surfaces by reflectivity. The built-up comparison film is mounted on the stage of a separate microscope also fitted with a vertical illuminator. The red cell membranes are matched against the step film with the aid of a comparison ocular, care being taken that the illuminators and objectives are themselves matched and that a single light source is used to illuminate both systems. With critical adjustment it is thus possible to determine average thicknesses with an accuracy of ± 10 AU. The method also gives information of an analytical sort, for the membranes can be leached with lipide solvents and the thickness of the protein residue determined. Knowing the thickness, hence volume, of the lipide and protein components, their weights can readily be estimated.

II. The ultrastructure of some typical protoplasmic systems. Tissue and cellular structures may be regarded as presenting a repetition in three dimensions of essentially two types of fundamental struc-

tures. These are the linear type represented by fibers and the lamellar type represented by interfacial films, membranes, sheaths, etc. Classification according to geometrical configuration rather than according to functional, anatomical or chemical relationships emphasizes the essential similarity in ultrastructure of all the members of each category. It will suffice for the present review, therefore, to present the evidence concerning the ultrastructure of only a few examples of each type, chosen because of their physiological significance and because they lend themselves well to investigation; the analysis of other fibrous and lamellar structures will, in general, follow closely along the same lines. More complete accounts may be found in the recent books and articles of Schmidt (162, 164, 166) and Frey-Wyssling (76).

A. Fibrous Structures. General remarks. Animal fibers may occur as large aggregates or bundles of smaller fibrils as in the case of supporting, elastic, and contractile tissues or in the form of microscopic, cellular structures such as spindle and astral fibers, chromosomes, cilia, etc. The data of greatest significance are those which reveal molecular configurations and these are obtained by the method of x-ray diffraction. Hence the most detailed information is that which has been obtained from tissues such as hair, tendon and muscle, which lend themselves readily to x-ray analysis. It is probable, however, that with certain precautions, the conclusions regarding ultrastructure reached from the analysis of these tissues may be applied to microscopic intracellular fibrils. This seems warranted in view of the close similarity between the polarization optical properties of tissue and cellular fibrils, detailed below.

Animal fibers may be considered according to two hierarchies of submicroscopic structure. The lower of these consists of the individual protein chain molecules and their relation with their neighboring chains; investigation of this aspect requires the x-ray diffraction method of analysis. The second hierarchy consists of aggregates of protein chains in the form of submicroscopic rodlets or micelles; information concerning such structures is best obtained from polarized light studies. A higher hierarchy would, of course, be the microscopically visible fibrillae themselves.

Protein fibers display mechanical, optical, electrical and magnetic anisotropies by virtue of the fact that they are constructed of polypeptide chain molecules which are oriented with long axes parallel to each other and to the long axis of the fiber. These chains are bonded to each other laterally by linkages between the terminal groups of the

side chains, and the physical and chemical properties of the fiber are determined largely by the relative stability of this lateral bonding (see 92, 20). If the protein is composed of amino acids which have long side chains with strongly polar terminal groups, the compactness, tensile strength, swelling, contractility, etc., will be very different than in the case of proteins composed of amino acids with short side chains having non-polar terminal groups. If the protein chains are closely packed and well integrated laterally by side linkages we have a typical mechanical tissue like hair. There is very little room for water in such fibers and there is practically no metabolism. If the protein contains a higher percentage of amino acids with long side chains having polar end groups, the fiber may still be fairly compact as in the case of tendon and connective tissue. In these the water content is much higher, though the metabolism is still low. More highly solvated protein fibers are those which characterize actively metabolizing, contractile systems such as muscle and intracellular fibrils. In these the configuration of the chains may be altered rapidly and reversibly thus producing contraction and extension. Highly solvated protein chains may also occur which are not integrated into definite, visible fibrils but which may readily be recruited into such systems by (enzymatic?) desolvation. Such a highly tenuous lattice is presumably the basis of the "cystoskeleton" postulated by Peters (127, 128, 122).

Since the molecular configuration and lateral bonding of the protein chains is most easily determined in compact tissues such as hair and tendon, the most detailed information has been obtained with these tissues. The data have been of great value in the interpretation of the structure of the physiologically more interesting fibers such as muscle.

Essentially two types of fibrous architecture are recognized at present, the keratinous and the collagenous types, both being composed of longitudinally oriented polypeptide chains bonded together by side-chain linkages.

The extensive work of Astbury on keratin structure has focussed attention upon the configuration of the chains in the long axis of the fiber. Under normal conditions the chains are not fully extended but are thrown into folds (α keratin), thus greatly reducing the overall length of the fiber. Under the influence of steam, α keratin may be stretched to approximately double its length, the folded chains being thus drawn out into the fully extended condition (β keratin). These changes may be made to occur reversibly. Under appropriate conditions, β keratin may be made to shorten to a length less than that of the original α

keratin (supercontracture). In this condition the chains are presumably more extensively folded than in the α condition.

Less is known about the folding of the chains in the case of collagenous fibers. Since collagen shows the phenomenon of supercontracture strikingly (98) it is assumed that the folding may become considerably greater than in the case of α keratin or perhaps even of supercontracted keratin. Astbury (1936) assumed that a meridional spacing of 2.84 AU observed by Katz (94, 95) represented the length of the amino acid residues along the chains; this would be the case if the chains are in a *cis-* rather than a *trans-* configuration as is the case for fully extended chains such as silk where the residue length is 3.5 AU. Clark and Schaad (47), confirmed by Champetier and Fauré-Fremiet (41) find that the length of the residue is 3.25 AU (from a meridional spacing three times this size), hence the chains are relatively little folded. The lateral distance between the chains (from side chain spacings) varies from about 11–17 AU depending upon the amount of water of solvation which is distributed between them.

A striking characteristic of collagen patterns, which may also occur in keratin patterns (Astbury, 9), though it has never been carefully examined in these, is the occurrence of meridional diffractions corresponding to very large periodicities (as large as 432 AU) *along the fiber axis* (46, 47, 215, 216, 55, 45). This suggests either that the fiber consists of parallel bundles of indefinitely extended polypeptide chains along which amino acid residues are regularly repeated with a master period as high as 300–400 AU, or alternatively, and perhaps more probably, that collagen is truly crystalline, being built up by a regular arrangement of very large molecules, the large spacings indicating the molecular dimensions. The latter possibility is suggested from the experiments of Wyckoff and Corey (215, 216) which show that dissolved collagen contains large particles which can be made to aggregate again to form a "tissue" giving patterns identical with those of normal tendon. Sedimentation data are not yet available which would indicate the size of the particles and whether or not they are monodisperse. The occurrence of such large aggregates or master periods is of considerable biological interest, particularly since similar structures are postulated as representing gene levels in the chromosomes. It is to be hoped that the structure underlying these large spacings in organized collagen and other fibrous proteins will be the subject of further detailed investigation.

1. Muscle Fibers. a. *X-ray diffraction evidence.* Early investiga-

tions of muscle structure by x-ray diffraction (85, 86, 25, 27, 206, 207, 208) brought out the fact that muscle contains longitudinally oriented chains separated laterally by side chains, in agreement with the Meyer-Mark (111, 112) concept of fiber structure. Impressed with the similarity between the long-range elastic and contractile phenomena of muscle and keratin and between the diffraction patterns of relaxed muscle and hair, Astbury undertook an extensive investigation of the meaning of these similarities. Although these investigations are not yet completed and only preliminary descriptions have been published (8, 10, further details were kindly communicated to the author privately), Astbury has come to the general conclusion that in resting muscle the protein chains are arranged in a folded configuration similar to that in α keratin, and that in contraction the chains are thrown into superfolds similar to the condition of supercontracture in hair. Evidence supporting these conclusions is given below.

These investigations have been enormously facilitated by: 1, the demonstration that the contractile fibrils are composed almost exclusively of a single type of protein, myosin, hence that the contractile and elastic properties must be due to intra- or intermolecular transformation of this protein; 2, by a fairly comprehensive survey of the physical, chemical, and optical properties of myosin (202, 203, 181, 182, 62, 119, 109, 114, 115, 125, 35, 36, 73, 14, 152, 154, 156, 161, 190, 191, 192, 105, 110, 187).

Boehm and Weber (27) obtained a fiber pattern from myosin threads formed by forcing myosin solutions into water through capillary jets. Astbury and Dickinson (11, 12, full paper in preparation) have obtained patterns from myosin films which had been somewhat stretched to produce orientation of the chains, which are so similar to those of α keratin as to be superimposable; if the films are stretched further the chains may be fully extended, and a pattern similar to β keratin is obtained. Strips of myosin film not only show extensibility properties similar to keratin fibers but exhibit also supercontraction and long range elasticity. Supercontracted myosin films, like elastoidin (40), give no new type of pattern although in maximal supercontraction the pattern is essentially a disoriented β pattern (two rings).

Since the evidence seems conclusive that contraction is due to changes in configuration of myosin "molecules" and since Astbury has demonstrated in rough outline the molecular changes which occur when oriented myosin chains undergo shortening *in vitro*, it remains but to show that the pattern of contracted muscle is similar to that of super-

contracted myosin films. The chief difficulty in such work is in arranging so that the muscle shall be radiated only while in the contracted state. Astbury (private communication) states that this desideratum has been approached in the case of slow muscles like those of Mytilus and that the pattern is essentially similar to that of supercontracted myosin films. From this it would appear that muscle contraction is due to a rapidly reversible superfolding of myosin chains. The evidence is perhaps not as crucial as might be desired since the pattern of the supercontracted state does not differ strikingly from the normal α pattern. It is impossible, therefore, on the grounds of the diffraction evidence alone to rule out mechanisms such as that postulated by Bernal (1937), according to which shortening in striated muscle is not due to molecular contraction but to transformation of straight, parallel chains (in the *A* bands) into a reverse spiral configuration (such as is assumed to occur normally in the *I* bands), by means of local desolvation.

Since the pioneering work of Brücke (34) and of Engelmann (64, 66) an extensive literature has accumulated regarding the polarized light analysis of muscle ultrastructure. This literature has been ably reviewed recently by Schmidt (162, 166). We shall present only a brief summary, therefore, of the more important points, stressing those aspects which are of general application in the analysis of protein fibers.

The contractile unit of muscle is the myofibril, which is now considered to be composed exclusively of myosin. Like most protein fibers it shows positive uniaxial birefringence with optic axis parallel to the long axis of the fiber. In smooth muscle the fibrils are uniformly birefringent throughout their length. In striated muscle the myosin fibrils are also continuous but the configuration of the molecular chains varies periodically along the length of the fiber, thus producing regions which show fairly strong positive birefringence (chains oriented parallel with the long axis) alternating with regions which show very weak positive birefringence (chains presumably greatly folded or disposed at angles to the axis). These constitute the *A* (for anisotropic, also called *Q* in the older literature) and the *I* (named on the erroneous assumption of complete isotropy) bands, respectively. The chains are not folded or crossed throughout the *I* bands, for at the level of the *Z* "membrane," the fibrils show positive crystalline birefringence (162), hence for very short distances the chains are again parallel. There is also a periodic difference either in the concentration of the myosin or in the lateral distance between the chains since the *A* regions have a

higher refractive index than the *I* regions and react differently with stains. The refractive index of the *A* regions is moreover not uniform but decreases towards the middle of the bands (Henson's zone). Since this complex structure is fairly typical of striated muscle generally, and since striation appears to have been developed in order to increase the velocity of contraction, clues to the mechanism of contraction have been sought in terms of rearrangements of the myosin chains in the alternately straight and folded or crossed chain regions. The recent speculation of Bernal (20), previously mentioned, is a stimulating attempt in this direction.

Muscle shows both positive crystalline and positive form birefringence, as was demonstrated first by Stübel (188, 189). This means that the oriented myosin chains are associated to form elongated aggregates in which the chains are arranged in semi-crystalline regularity. The existence of such coherent particles is strongly indicated by the fact that solutions of myosin show stream birefringence (118, 119, 26). Weber (203) found that fibers made by forcing myosin into water through a capillary jet showed form birefringence closely similar to that of muscle. From these data, together with approximate figures for the volume of the myosin micelle (187, 202, 203) the dimensions of the micelles and their average separation in the muscle fiber were estimated. A similar analysis of the configurations of the micelles has recently been made by Fischer (73, 74, 75) for smooth muscle both in the resting and in the contracted states. He found that the birefringence of smooth muscle depends on the length of the muscle, increasing as the muscle is extended. This was confirmed by Bozler and Cottrell (31). Using a method which avoids the necessity of measuring the muscle thickness accurately Bozler and Cottrell found that the birefringence is proportional approximately to the square root of the length of the muscle. They suggest that as the muscle is extended more myosin molecules become oriented in monomolecular layers at surfaces within the fibers. The square root relationship has been confirmed recently by Fischer (75).

It has long been known that the birefringence of striated muscle decreases during contraction (200, 60, 61). v. Muralt (117) studied this optical negative variation in the case of isometric contraction of striated muscle, using a sensitive method of continuous recording. He observed a decrease of some 40 per cent, the curve showing two minima which could not well be correlated either with mechanical or with energy changes. He assumed the decrease was due to reduced crystalline birefringence,

as might have been expected from the Meyer-Mark theory of contraction. The significance of the optical negative variation has become even more uncertain as a result of recent experiments with smooth muscle. Fischer (73) finds that if the muscle is not greatly extended, contraction results in decrease in birefringence, agreeing with v. Muralt. With stretched muscle, however, this decrease may not occur; in fact, with greatly extended muscle, contraction produces an increase in birefringence. This was attributed to a photoelastic effect resulting from stretching. Bozler and Cottrell (31) claim that the photoelastic effect can be avoided by destroying the ganglion which is attached to the muscle; the muscle can then be greatly extended without development of tension. Under these conditions isometric contraction results in no change in birefringence. Applying the method to frog sartorius muscle, the decrease found by v. Muralt could be obtained only under special conditions of tension. Under other conditions little change could be observed. They believe that birefringence is not fundamentally associated with tension development or with activity in general but rather merely with the length of the muscle; if the muscle is allowed to shorten, then birefringence will decrease simply because the muscle becomes shorter, the birefringence varying as the square root of the muscle length. Confirmation of these experiments is highly desirable since the conclusions seem to disagree with the view which has become generally accepted, that tension development and shortening involve a contraction of myosin chains in the Meyer and Mark or Astbury sense or a rearrangement of chains in the micelle according to certain geometrical schemes, such as that of Bernal (20); in either case contraction should be associated with a decrease in birefringence.

2. Cellular Fibers. It has long been known that contractile cellular fibers such as cilia, flagellae, axopodia, filopodia and myonemes of protozoa, sperm tails, etc., are similar to myofibrils in showing positive uniaxial birefringence with the optic axis parallel to the fiber axis. Though it is impossible to obtain x-ray evidence of the molecular structure of these microscopic fibers, it is reasonable to suppose that they are composed of oriented protein chains, as in the case of muscle fibers, since the polarization optical properties are similar to those of muscle.

The optical properties of cilia have been examined extensively. Cilia show both positive crystalline and positive form birefringence (107, 81), hence contain elongated particles oriented parallel with the axis of the cilia. Considerable lipide is also present and under suitable

conditions the sign of birefringence may be reversed to negative due to lipide molecules adsorbed with their paraffin chains oriented perpendicular to the protein chains (143, 78). It seems probable that at least a portion of this lipide is so oriented under normal conditions, as may be judged from the experiments of Göthlin (81) and recent interpretations of Bear and Schmitt (16). The intracellular fibrillar continuations of cilia also show positive birefringence and striking dichroism (65, 146). Even the basal granules which unite the cilia with the intracellular fibrils show strong gold dichroism. This high degree of molecular organization of the ciliary mechanism is of importance not only for contractility but doubtless also for the neuroid regulation which causes all the cilia in a cell and in the epithelium to beat in a concerted metachronous manner.

Even through a fibril may be fairly birefringent, if it is extremely small in cross section, the phase difference or retardation will be proportionately small, hence it will be impossible to detect the birefringence unless the compensator is very sensitive and the illumination is intense. Since the sensitive Köhler rotating plate compensator, having total retardations of only one-tenth to one-thirtieth of a wave length, has come into general use in biological work, birefringence has been detected in extremely thin cellular fibrils. Among these are the spindle and astral fibers and chromosomes of dividing cells.

Spindle and astral fibrils show positive uniaxial birefringence with optic axis longitudinal. Runnström (139) observed this in a plant cell, *Fritillaria imperialis*, and claimed also to have demonstrated the presence of lipides associated with protein chains (reversal of birefringence after immersion in glycerine). Schmidt (157, 158) observed the positive birefringence of spindle and astral fibers in living sea urchin (*Psammechinus*) eggs and states that, like typical contractile fibers, they show positive crystalline and form birefringence. Similar optical properties were observed in the case of neurofibrils in fresh nerve cells (42) and axons (18, 19). Although the fibrils cannot be observed in the fresh nerve cells with the ordinary microscope, the picture in the polarizing microscope is that to be expected if fibrils existed in the configurations shown in fixed preparations and if the fibrils are positively birefringent with respect to their long axes. It would seem that if an oriented protein cytoskeleton, such as has been postulated by Peters (127, 128), actually exists in cells its presence should be detectable with the most refined polarization optical methods. Something of this sort may underlie the observations of Moore and Miller (116) on the cytoplasm of centrifuged sea urchin eggs.

Chromosomes represent a type of fiber of great interest and biological importance. Cytogenetic studies having demonstrated the existence of determiners of heredity and development in chromosomes, and having even arranged the relative positions of the various genes along the chromosomes according to linear maps, it is highly important that the molecular architecture of the chromosomes be investigated in order to furnish a physical basis for gene structure. While the electron microscope may eventually furnish the most conclusive evidence, polarized light studies have revealed significant facts and x-ray diffraction experiments on artificial models have contributed fundamental information.

Early attempts to discover birefringence in chromosomes were unsuccessful (136, 69, 67). It has long been known, however, that sperm nuclei are birefringent (63, 199, 64) and since sperm nuclei contain highly condensed chromosome material these optical properties suggested the lines along which chromosome structure was subsequently to be analyzed. The most detailed information on this subject is due to Schmidt (144, 145, 147, 148, 150, 151, 162).

The birefringence of sperm heads depends on the relative degree of solvation of the nucleoprotein material, hence varies with different species and with the stage of development. Typical long slender sperm heads show negative uniaxial crystalline birefringence with optic axes parallel to the long axes of the sperm heads. In earlier, less desolvated stages the sperm may show very weak positive birefringence. These facts are explained by Schmidt (149, 151) on the basis of experiments with models consisting of stretched nucleic acid gels or of fibers made by forcing concentrated solutions of α thymonucleic acid through capillary jets into alcohol. The alcohol-dehydrated fibers show negative uniaxial crystalline birefringence like desolvated sperm heads. Before desolvation the fibers may show weak positive form birefringence, indicating that the particles are rod shaped with long axes parallel to the axis of the fiber. Chromatin may therefore show either weak positive or relatively strong negative birefringence depending on whether it is relatively solvated or desolvated.

In the relatively transparent eggs of *Psammechinus*, Schmidt (158, 159) recently observed striking birefringence of chromosomes in living, dividing eggs. The birefringence develops during metaphase, is maximum during anaphase, decreases in telophase and vanishes in late telophase. The sign of birefringence is positive with respect to the axis of the spindle. This is explained by Schmidt as follows. In the interkinetic nucleus the chromatin micelles are highly solvated and relatively disoriented, hence show no birefringence. With the onset of

cell division the micelles become oriented with long axes parallel to the axis of the chromosomes and in metaphase and anaphase the desolvation is sufficient to produce marked rodlet form birefringence, though not sufficient to allow the (negative) crystalline birefringence of the chromatin micelles to overcome the (positive) form birefringence.

More recently, important information has come from a study of the giant banded chromosomes of insect salivary glands (198, 160, 165). In these unusual, highly expanded chromosomes, it had been demonstrated by Caspersson (38) that the nucleic acid is located in the chromatic bands, the achromatic bands being composed of protein. Schmidt finds that the chromatic bands, when desolvated in alcohol, show crystalline birefringence which is negative with respect to the long axis of the chromosome. This he interprets as due to the presence of oriented nucleic acid micelles. From Caspersson's data one might expect the achromatic bands to show positive birefringence typical of protein fibers, but this has not thus far been demonstrated.

Recently it has been found that thymonucleic acid occurs in solution as thin rodlet particles with a length-to-thickness ratio of about 300:1 and that these particles show strong negative fluxional birefringence (179). The micelles or "molecules" have a weight of $5 - 10 \times 10^5$ (180). Films of thymonucleate stretched 250 per cent give x-ray diffraction patterns typical of fibers, the most striking reflection along the fiber axis occurring at 3.3 AU. Since this corresponds fairly closely with the length of the amino acid residue in proteins in which the chains are fully extended (β condition), Astbury and Bell (13) suggest that in fibers the nucleic acid is anchored laterally to the protein side chains through terminal linkages of the nucleotide groups, the latter projecting perpendicularly to the axis of the nucleic acid micelles. This view, which is inconsistent with that suggested by Wrinch (209–212), is confirmed by the fact that the fibrous compound of nucleic acid with the protein clupein gives sharp diffraction patterns which resemble those of nucleic acid itself (Astbury, private communication). Since the protein primary valence chains of chromosomes, through their side chains, fit on automatically down the sides of the long nucleic acid micelles it seems probable that the nucleic acid may play an important rôle in the synthesis of chromatin material by establishing a pattern according to which new protein chains may be laid down. This suggestion is supported by direct observations on the disposition of nucleic acid in the chromosomes under certain genetically abnormal conditions (39). Since the different nucleotides may occur along the nucleic acid

micelle according to varying patterns, the importance of the nucleic acid component in determining specific genic patterns is obvious.

B. *Lamellar (Membranous) Structures.* The macroscopic and microscopic appearance of membranes and films suggests the shapes of their submicroscopic building-stones, the latter consisting of layers of molecules oriented in planes parallel to the surface of the membrane. Such structures may be composed of a few molecular layers only, as in the case of thin interfacial films, or they may be composed of dozens or hundreds of layers, as in the case of microscopically visible membranes or of sheaths which may be several microns in thickness. The molecular architecture is probably fundamentally similar regardless of the relative thickness. The most favorable case in which to determine the fundamental ultrastructure of such systems is the nerve myelin sheath, not only because it represents the most highly developed lipide-protein lamellar system but because it lends itself readily to examination by x-ray diffraction, thus permitting an investigation of the details of molecular structure. Because of their microscopic size most cellular membranes can be investigated only by the polarized light method. The analysis of myelin sheath structure illustrates in a striking way the advantages of the use of both methods in a complementary fashion. Moreover, the information regarding the general properties of lipide-protein lamellar systems which is obtained from this complex system is useful in interpreting the structure of thinner cellular membranes which can be studied only by polarized light methods.

1. The Nerve Myelin Sheath. The structure of the nerve axon sheath has been discussed at length in several recent reviews (167, 168, 162, 171). In the present section we shall present a brief summary of this information without attempting to include details which are chiefly of incidental or of historical significance.

Considering first the polarization optical properties of the sheath, we find that, like tubular myelin forms produced by the action of water on the sheath lipides, the sheath of a fresh fiber shows positive uniaxial birefringence with optic axes directed radially. This is a property of the lipides since extraction with alcohol abolishes this birefringence and leaves a protein residue which exhibits weak negative uniaxial birefringence with optic axes also radially directed. Immersing such alcohol-extracted fibers in solutions of varying refractive index shows that this protein birefringence is form birefringence. Since the sign of birefringence is negative with respect to the direction of the optic axis, the system may be assumed to be constructed of ultramicroscopic protein

lamellae oriented in planes parallel to the surface of the sheath (113, 155, 43). The normal sheath, therefore, presents a unified system consisting of concentric layers of protein interspersed with layers of lipide, the thickness of the layers being considerably less than the wave length of light. Since the lipides are long chain molecules which are optically positive with respect to their long axes it may be inferred that the lipide molecules are oriented with their chains perpendicular to the plane of the layers, i.e., radially in the sheath.

Because of the radial orientation of the optic axes the birefringence of the sheath cannot be measured in the customary manner since the optically effective thickness is no obvious function of fiber or sheath dimensions. To make such measurements possible an expression was derived (15) relating birefringence, optical retardation (measured with a compensator), and axis cylinder and fiber diameters. The birefringence of the sheaths of frog *A* fibers thus calculated was found to be 0.011 (170). This relatively high value indicates that the lipide molecules are well oriented in the radial direction within the layers of the sheath.

More detailed information about the molecular configuration of the sheath was obtained by x-ray diffraction studies (169, 171). The diffraction patterns of fresh nerves appear to be due exclusively to the myelin sheath and are essentially similar in all vertebrate nerves so far examined. The most prominent diffractions consist of a meridionally accentuated ring corresponding to a spacing of 4.7 AU, a very faint meriodonal reflection at about 9.4 AU, and a series of equatorial spots representing orders of a fundamental spacing of 170–180 AU, the period varying somewhat with different types of nerves.

In the light of the polarization optical analysis these patterns have been interpreted as follows. The identity period of approximately 171 AU is considered to represent the thickness (in the radial direction) of the concentric lamellae. Pure lipides, or mixtures of lipides as found in nerve, give spacings to be expected of double layers of molecules and in the average these spacings are less than half that of the nerve spacing. The unit lamella of the sheath is therefore assumed to contain two double layers of mixed lipide molecules oriented with long paraffin chains radial, bounded by layers of protein, probably one, or at the most only a few molecules, in thickness. The polar groups of each molecular layer are presumably in apposition at the boundaries forming "phases" which are alternately predominantly aqueous and predominantly hydrocarbon.

The 4.7 and 9.4 AU meridional diffractions are thought to represent

the average distance between the lipide chains and molecules, respectively, in the tangential direction, e.g., within the planes of the layers. In these planes the lipide molecules are distributed in a random fashion as in a liquid, though the packing is fairly close.

The polarized light and x-ray diffraction studies, therefore, lead to the conclusion that the myelin sheath, like tubular lipide myelin forms, is composed of cylindrical smectic fluid crystalline layers wrapped concentrically, the sheath being more complex than myelin forms by virtue of the inclusion between the lipide layers or sheets of neurokeratinogenic protein material; this bonding of the lipide molecules on a possibly well organized protein manifold contributes to the stability of the myelin sheath as compared with that of myelin forms.

Although only heavily myelinated fibers have been studied in detail by x-ray diffraction, the polarized light analysis has been extended to include small as well as large fibers, invertebrate as well as vertebrate, and the above general scheme has been found to be of general application over the whole range. Though, as in the case of the so-called unmyelinated fibers, the lipide concentration may be very low, its presence and typical orientation can usually be detected by immersing the fibers in strongly polar media (e.g., glucose, glycerine, salts, etc.) of high refractive index; this abolishes or reduces the protein form birefringence and unmasks the lipide birefringence which is of opposite sign (16, 43). This technique has in fact been used to discover and localize the axon sheath in the case of fibers which are surrounded by many layers of connective tissue which are indistinguishable histochemically from the true axon sheath (17).

As yet no changes in molecular orientation in the sheath have been demonstrated to be associated with the passage of the nerve impulse. This is perhaps not unexpected in view of the very small energy changes associated with the initial electrical phenomena (87) although the large changes in resistance recently reported (52) suggest some alteration of molecular structure either in the sheath as a whole or in some critical interface in the sheath. In any case, the ultrastructure and chemical composition of the sheath must influence physiological properties such as conduction-velocity, since comparisons over a wide range of fiber types show that incorporation in the sheath of large amounts of oriented lipide favors rapid conduction; in the absence of such oriented lipide, rapid conduction is achieved only by tremendous increase of fiber diameter (16, 171, 133).

2. The Cell and Nuclear Membranes. A large body of evidence from

investigations of permeability (89, 90, 88, 33, 132), tensions at cell and protoplasmic interfaces (84), electrical impedance of cell membranes (77, 50–53), direct chemical analysis of cell membranes (80, 68, 193, 194, 30), studies of models of membranes (56, 57), etc., indicates clearly that the plasma membrane is a thin lipide-protein film, probably only a few molecular layers in thickness. It is of considerable physiological importance to know the ultrastructure of this film, particularly with regard to whether it is to be considered a mosaic of lipide and protein constituents or whether it has a layered structure like the myelin sheath, with alternate hydrocarbon (lipide) and aqueous (protein) phases.

The ultrastructure of the plasma membrane may be investigated very conveniently in the case of the mammalian erythrocyte. Under appropriate conditions of hemolysis the hemoglobin may be removed from the cells leaving merely the limiting envelope surrounding an aqueous solution. The polarization optical properties of this envelope have been found to correspond closely with those of the axon sheath of invertebrate nerve fibers: lipide components contribute positive uniaxial crystalline birefringence with optic axes radial; protein components contribute negative uniaxial form birefringence, also with optic axes radial (172). Hence the membrane is a layered structure containing lipide molecules with long paraffin chains oriented perpendicular to the surface and with protein leaflets oriented with long axes tangential, i.e., with flat surfaces parallel to the surface of the membrane. The polarized light studies do not reveal whether, as in the myelin sheath, the lipide and protein components are arranged in separate and continuous layers or whether the structure is essentially that of a mosaic.

Considering the red cell membrane as a Wiener mixed body it is possible to calculate the ratio of lipide to protein constituents in the membrane by volume and by weight (173). The ratio of partial *volumes* thus determined from optical properties is 2.0. This is in very good agreement with the value 1.7 determined directly by the interference method described on p. 282. It has been found (174) that the total thickness of the rabbit red cell membrane is about 160 AU and that of this thickness about 100 AU are occupied by lipide molecules, leaving about 60 AU for the protein constituents, the ratio of partial volumes being approximately 1.7. The ratio of partial *weights* calculated from the Wiener relation is approximately 1.2. This is in fair agreement with data obtained from direct analysis of stromata (93, 131, 68), though such analyses are notoriously unreliable with respect to the protein figures because of the difficulty of removing completely the large quantities of hemoglobin from the stromatin suspension.

The relative difficulty encountered in leaching the lipide from the red cell membranes, particularly after the membranes had been dried is doubtless due to the restraining influence of the protein material and strongly suggests that the latter occurs as extensive, though perhaps not necessarily continuous, layers between the oriented lipide molecules. If the protein occurred as small leaflet patches distributed between the lipide molecules according to a mosaic, leaching would almost certainly take place very much more rapidly. This seems reasonable from the results of Langmuir, Schaefer and Wrinch (102), working with built-up films of stearate and protein.

Polarization optical effects similar to those described for the red cell envelope have been observed also in the cell membranes of marine eggs (138, 139) and of nerve cells (42). In fresh nerve cells the nuclear membrane shows a negative polarization cross indicative of the presence of protein lamellae; if oriented lipide is present its concentration is too low to be detected even by the sensitive optical method (42). It is possible, however, in certain other cells to demonstrate the presence of oriented lipide interspersed in the protein matrix of the nuclear membrane. It seems probable that the structure typical of cell and nuclear membranes as well as protoplasmic membranes and interfaces generally is that of a series of protein leaflets lying in planes parallel to the surface of the interface, possibly with a certain amount of interconnection between leaflets, and containing a variable quantity of lipide molecules oriented with long paraffin chains perpendicular to the surfaces of the protein leaflets.

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